Clinical Chemistry Trainee Council
Pearls of Laboratory Medicine
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TITLE: Automated Hematology

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Hello, my name is Lauren King. I am a hematopathology fellow at Houston Methodist Hospital in Houston, Texas. Welcome to this Pearl of Laboratory Medicine on "Automated Hematology." The hematology analyzer is a vital part of every clinical laboratory and the results provided change the course of therapy for many patients. Understanding the methodology, and thus the limitations, of the equipment is necessary to provide the best care to our patients.

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The complete blood count or "CBC" measures the cellular components of the blood. There are many different indications for performing a CBC. The number, size, shape, and hemoglobin concentration of erythrocytes determine the presence and often the cause of anemia. The leukocyte count is important in the diagnosis of infection, hematologic diseases such as leukemia, and immune status disorders. The platelet count provides analysis of one of the body's mechanisms of hemostasis.

Historically, laboratory personnel performed manual complete blood counts using dilutions and a hemocytometer. Thankfully, instruments are now automated, using a variety of methodologies that will be discussed. This automation provides fast throughput with accurate, reliable, and precise results.

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Parameters that are measured in a CBC can be divided into three categories: Erythrocytes, leukocytes, and platelets. We will discuss how each of these values is obtained throughout this session.

The erythrocyte values include: the red blood cell count (RBC), the hemoglobin concentration, hematocrit, the mean corpuscular volume (MCV), the mean cell hemoglobin (MCH), the mean corpuscular hemoglobin concentration (MCHC), and the red cell distribution width (RDW). The reticulocyte, or immature red cell, count is also often included.

Leukocyte values generally include a total number of white blood cells, expressed as a function of 10^3 per microliter or cubic millimeter, as well as a differential including neutrophils, lymphocytes, monocytes, eosinophils, basophils. The differential can be expressed in absolute numbers and/or percentages of the total white blood cell count.

Platelet values commonly include the platelet count and mean platelet volume, also known as the MPV.

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Each analyzer employs different combinations of methodologies to produce results and specific computer software to aid in analysis; however, the basic principles used remain the same.

The electrical impedance method of measurement is based on the change in resistance that occurs when a red cell, which has low conductivity, passes through an electrical field. As the conductance across the field changes, a pulse is produced. The frequency of the pulse provides the cell number; the amplitude of that pulse is proportional to the cell's volume. This provides a count and a size measurement for each cell passing through an electrically charged aperture. This method is also known as resistive pulse or the Coulter principle.

Light scatter technology is the second method that is frequently employed by analyzers. As a cell passes through a beam of optical light or a laser, it scatters the light, which can then be detected using specifically positioned photodetectors. The interruption of light allows the cells to be counted. The scatter of lights allows us to learn about the cell structure. The light that is scattered in a forward direction is a measure of cell size, while the light scattered to the side is a measure of cell complexity or granularity.

Fluorescent dyes can also be added to cells to aid in identification of populations. In these techniques, agents are used to perforate cell membranes so the fluorescent dyes can bind to RNA and DNA. The fluorescent intensity can then be used as an additional parameter to separate cell populations.

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Electrical impedance is the most common method used to measure the erythrocytes, although some instruments do use light scatter or a combination of both techniques. As the number of cells is counted and the size of each is measured, a histogram is formed of the results, where the X-axis is size and Y-axis is number of events. A "window" is set for erythrocytes, so that pulses which are too small or too large will not alter the erythrocyte count.

An average volume is calculated from all events within the erythrocyte window. This is the mean corpuscular volume, or MCV, which is used clinically to define anemia as microcytic, normocytic, or macrocytic.

The width of this curve is then translated into the numeric value of the red cell distribution width, or RDW. The RDW can be expressed as either a coefficient of variation or standard deviation. The CV bases the parameter on the mean corpuscular volume, while the SD directly measures the erythrocyte population and compares the cells to themselves. The RDW is a measure of the variability present in an erythrocyte population.

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The histogram curve also demonstrates graphically the proportion of cells of each size. For example, a patient with macrocytic anemia, or large red cells, would have a histogram that is shifted to the right, whereas a patient who has a distinct mix of two sizes of red cells may have a curve with bimodal peaks. This situation could occur in a patient with microcytic anemia who is transfused with normal sized erythrocytes.

As cells are measured by the electrical pulse they generate, it stands to reason that any disruption in cell size that would place the RBCs outside of the window for analysis will interfere with the results. For example, in a patient with numerous schistocytes, or red blood cell fragments, the small pieces of red cell may not be large enough to fall within the analytical range, and thus, a falsely decreased red cell count may be reported. Similarly, if red blood cells do not flow through the aperture one at a time and instead stick together, they may register as events which are "too large" to be red blood cells. The analytical software that accompanies the analyzers attempts to correct for occasional instances where the red cells may pass through together; however, if it happens too many times, the results may be affected.

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The other vital part of erythrocyte analysis is measurement of hemoglobin.

There are two main methods used to measure the amount of hemoglobin contained in a sample of blood, although several analyzers employ manufacturer specific methods. The gold standard is the hemiglobincyanide method.

In the classic hemiglobincyanide method, blood is diluted in a solution of potassium ferricyanide and potassium cyanide. The hemoglobin is oxidized to methemoglobin, and then converted to hemiglobincyanide by addition of cyanide ions. Light absorption is measured at 540nm, and then compared to a curve obtained from a standard solution to calculate the hemoglobin concentration of the sample. One major downside to this method is the requirement for the toxic cyanide reagent.
A second method is the sodium lauryl sulfate method where surfactants lyse the erythrocytes and release hemoglobin. The sodium lauryl sulfate then converts ferrous iron to ferric iron, forming methemoglobin, which combines with SLS to form an SLS-hemichrome molecule. Measuring the absorbance of this substance at 555nm can then determine the hemoglobin concentration (g/dL). This method is more rapid than the traditional hemoglobin-cyanide method and does not require the dangerous cyanide compound.

It should be mentioned that since these methods depend on light absorption, any alterations to the solution that increase light scattering and cause the blood to appear to be absorbing more light will alter the results. These causes include increased turbidity due to increased plasma proteins, hyperlipemia, or even high white counts. Additionally, sulfhemoglobin, which forms during oxidative hemolysis and cannot bind to oxygen, is not measured by these methods.

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We've talked about four parameters directly measured by the analyzer: the red blood cell count, the mean corpuscular volume, the hemoglobin concentration, and the red cell distribution width.

The other parameters that make up the CBC are then calculated by the computer result, based on the measured data.

- The hematocrit is calculated by multiplying the MCV by the RBC count, then dividing by 10.
- The mean cell hemoglobin is calculated by dividing the hemoglobin concentration by the red blood cell count, and multiplying by 10.
- The mean corpuscular hemoglobin concentration is calculated from a ratio of hemoglobin to hematocrit, where hemoglobin is divided by hematocrit and the result is multiplied by 100.

The box on the right side illustrates an interesting scenario that relates to these measured and calculated values. In cold agglutinin disease, where the red cells stick together at room temperature, the MCV can be falsely elevated and the RBC count can be falsely decreased as cells travel through the aperture in a non-single-file manner. Since all other parameters are calculated from these important measurements, almost the entire CBC is inaccurate. The only value that is not affected is the hemoglobin, since the cells are lysed in order to undergo the chemical reaction for analysis.

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Reticulocytes are immature red blood cells that have lost their nucleus but still contain RNA. The number of these cells present can be used for determining response to iron or erythropoietin therapy, measuring bone marrow engraftment following transplant, and for evaluating causes of anemia, such as hypoproliferative bone marrow conditions.

Historically, manual reticulocyte counts were performed, where the laboratory personnel subjected samples to methylene blue or brilliant cresyl blue, and then counted cells that contained precipitated RNA complexes, which are seen as filamentous or granular erythrocyte inclusions.

In modern automated hematology, various methods are employed by analyzers to measure the reticulocyte count. These methods involve new methylene blue or fluorescent dyes that penetrate the blood cells and bind to RNA and DNA in the nucleated cells. Then forward scatter and fluorescence intensity for each cell is measured, and the scatter plot can be used to determine populations of mature erythrocytes and reticulocytes. The intensity of intracellular staining or fluorescence is used in some analyzers to determine the maturity of the reticulocytes.

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The measurement of leukocytes commonly involves the use of light scatter technology. In most analyzers, a portion of the blood sample is diluted and then the red cells are lysed. As the leukocytes are passed through the light beam, each population's forward and side scatter properties separate the cells on a scatterplot, which can then be translated numerically into a white blood cell count and differential.

Fluorescent dye aids in the separation of the populations and the differentiation of more cell types. When the red cells are lysed, there are also "holes" punched in the nucleated cells, which allows fluorescent dye to bind to RNA and DNA. The fluorescent intensity separates lymphocytes, monocytes, and eosinophils; however, neutrophils and basophils remain virtually indistinguishable.

A second portion of the blood sample is subjected to a stronger lyse agent that destroys all erythrocytes and leukocytes, except for the hardy basophils. The light scatter of this portion easily separates the basophils from all other destroyed cells, including the neutrophils, and thus, the neutrophil population can be determined by subtracting the basophils from the first histogram. Therefore, all five populations of white blood cells in a blood sample can be distinguished from one another.

Many systems also measure leukocytes by impedance simultaneously, generating a count that is measured in a similar fashion to the erythrocytes and platelets. The expected "window" that leukocytes would fall in is, of course, larger than that of erythrocytes or platelets. The impedance results can be used in correlation with the light scatter results to ensure accuracy.

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Some modern analyzers also allow enumeration of the immature granulocyte population, which includes metamyelocytes, myelocytes, promyelocytes, and in some instruments, blasts.

On some instruments, a separate channel on the analyzer provides this measure. Reagents that act on lipid membranes are used: the immature granulocytes have less lipid in their membrane than more mature forms, and thus the immature forms are left intact while the more mature forms are lysed. Direct current and radio frequency, which can measure internal density of cells, are used to enumerate the immature granulocytes which remain intact.

Other instruments calculate an immature granulocyte population by using specially designed software with the white blood cell scatterplots that have been generated as previously outlined.

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One special consideration in the analysis of leukocytes and erythrocytes is the presence of nucleated red blood cells. The importance of detecting the nucleated red cells is two-fold: first, the presence of a nucleated red cell population in a peripheral blood sample is abnormal, and second, the leukocyte count is falsely elevated by their presence. Computer software can either flag the results for a manual correction or use an algorithm to subtract the nucleated reds from the leukocyte count.

In many analyzers, nucleated red blood cells are part of the scatterplot created by light scatter. When a stereotypical population occurs on the scatterplot, the software examines the histogram produced by impedance studies. If there are cells falling to the left of where lymphocytes would be expected to occur, then the presence of nucleated red blood cells is likely. At least one other instrument uses fluorescent dye in combination with a lyse agent to remove the cytoplasm from the nucleated erythrocytes, thus forming a stereotypical fluorescent intensity for the population.

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Platelets can also be counted using impedance technology, with a range of much smaller sizes expected. Again, results of the volume (based on pulse amplitude) and number are plotted on a histogram, and a mean platelet volume, or MPV, and platelet distribution width can be obtained.

One shortcoming in the platelet count is the introduction of spurious increases or decreases. Platelet clumping, which can be a function of the anticoagulant used in the sample or physiologic properties of the patient, can falsely lower the platelet count, as the impedance pulses will be generated above the threshold set for platelets as clumps pass through. The same scenario can be seen in patients with large platelet size, such as May-Hegglin anomaly or Bernard-Soulier syndrome.

Spurious increases in platelet count can result from the presence of erythrocyte fragments. One scenario where this could be particularly dangerous is in the presence of Thrombotic Thrombocytopenic Purpura (TTP), where patients have microangiopathic hemolytic anemia resulting in schistocytes and thrombocytopenia. If the schistocytes were to be counted as platelets, then the thrombocytopenia may not be recognized, and the patient may be thought to just have anemia, leading to a delay in treatment.

One way to improve accuracy of enumerating platelets is evaluation by light scatter, either in addition to the impedance method automatically, or as a reflex when abnormalities are present. Some analyzers even offer fluorescent platelet counting, with a similar methodology to that employed in leukocyte differentials. Integrating these results can improve the platelet count accuracy by including large platelets while excluding red cell fragments, microcytic red cells, or red cell ghosts.

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When it comes to reporting results, most laboratories have their hematology analyzers interfaced with their laboratory information system in order to provide fast turn-around times and minimize human data input, which can introduce post-analytical error. Many of these systems will allow release of normal results as well as abnormal results that fulfill certain criteria.

However, it is worth mentioning special situations that necessitate manual review of a sample. As the analyzer correlates the results obtained using its combination of methodologies and computer software, there will be occasional cases which cannot be resolved and result in "flags." Each analyzer has a different algorithm of steps to take for reconciling discrepant results; however, the ultimate decision regarding unusual findings rests in the hands of the human operators.

Manual review of a prepared blood smear can often differentiate between true critical or serious results, and analytical difficulties. Each laboratory also sets certain criteria, based on their patient population, which will always necessitate manual review by a medical technologist and/or review by a pathologist. These parameters may include extremely high or low cell counts, immature cells such as blasts, evidence of red cell fragments, or other parameters the lab sees fit.

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In conclusion, here are some final take home points worth mentioning:

- Methodologies vary from analyzer to analyzer, but the most basic two principles are electrical impedance technology and light-scatter technology.
- The measured parameters of erythrocytes are hemoglobin, MCV, RBC count, and RDW. The other red cell parameters are calculated from these results.
- Nucleated red blood cells, schistocytes, and large platelets can all commonly interfere with automated hematology analyzers; however, modern instruments contain software and techniques to minimize their effect.

Slide 16: References

Slide 17: Disclosures

Slide 18: Thank You from www.TraineeCouncil.org

Thank you for joining me on this Pearl of Laboratory Medicine on "Automated Hematology." My name is Lauren King.