INTRODUCTION

Peripheral blood smear (PBS) examination is a very important tool used in diagnosis and follow up of abnormal results on a complete blood count (CBC) to evaluate different types of blood cells. It helps to diagnose and monitor numerous conditions that affect blood cell populations ***(Bain; 2011).***

The routine microscopic examination of a well spread, leishmann-stained PBS for RBCs morphology presents a wealth of information regarding red cell size, shape, inclusions and hemoglobin content that is basic and fundamental in hematology testing ***(Kim et al., 2003).***

During the past three decades, a number of automated hematology analyzers have been developed; most of which directly measure two RBCs parameters, RBCs count and mean corpuscular volume (MCV). The rest of the RBCs indices are calculated from RBC, MCV and hemoglobin (Hb) ***(Buttarello and Plebani, 2008).***

Nowadays, hematology automation offers more than CBC testing. There are four immature RBC parameters that can be automatically reported with every CBC to provide the information needed for the physician to assess the state of erythropoiesis. These parameters are number of circulating reticulocytes (RET), immature reticulocyte fraction (IRF), reticulocyte Hb content (CHr) and nucleated RBCs (nRBC). All these four parameters are widely

available now and can be useful to physicians in diagnosis of iron deficiency anemia because the parameters are direct measures that reflect the iron available for Hb synthesis ***(VanWyck, 2010).***

Owing to the fact that manual microscopic examination of the blood smear is subjective, time consuming and quite misleading impressions can be drawn from inadequately prepared smears, a number of automated hematology analyzers have been developed to manage heavy workload. But due to the incompleteness of accurate morphological information on individual RBCs from these instruments , 5-10 % of samples in hematology laboratories undergo smear review for abnormal RBCs morphology, from where comes the importance of studying the relationship between RBCs morphology reporting and the results of automated RBCs parameters from hematology analyzers ***(Gulali et al., 2013).***

With the help of different statistical tests and algorithms, the relationship between the complex data obtained from hematology analyzers regarding RBCs parameters and blood smear examination results can be analyzed in order to create a practical guideline that may help predicting RBCs morphology from hematological parameters of 4 data sets (MCV, MCH, HCT and RBCs) ***(Saichanma et al., 2014).***

Aim of the Work

The aim of the present study is to investigate the relationship between red blood cell morphology reporting by smear review and RBCs parameters obtained from hematology analyzers and whether this could help in predicting RBCs morphological abnormalities as good as individual subjective reporting.

Chapter 1: Red Blood Cells Morphology

Erythropoiesis is defined as the pathway producing mature red blood cells (RBCs) from hematopoietic stem cells. This process includes several steps restricting differentiation and proliferation of cells which undergo this erythroid program, depending on sequential and specific erythroid gene expression. Erythropoiesis is regulated by combined effects of microenvironment and growth factors that promote survival, proliferation, and/or differentiation of erythroid progenitors and nuclear factors that regulate transcription of genes involved in survival and establishment of the erythroid phenotype ***(Dessypris and Sawyer, 2009)).***

There are several control mechanisms regulating RBCs production. Of them, the glycoprotein hormone Erythropoietin (EPO) has been established as the major humoral regulator. Erythropoiesis involves a great variety and number of cells at different stages of maturation starting with the first stem cell progeny which are colony forming unit granulocyte, erythroid, monocyte and megakaryocyte (GFU_{GEMM}) committed to erythroid differentiation and ending with the mature circulating RBCs ***(Cantor and Orkin, 2002).***

Completely mature RBC is a non-nucleated biconcave disc. The single pronormoblast gives rise to 16 mature RBCs. Normally normoblasts are not seen in peripheral blood except in extramedullary erythropoiesis occurring outside the bone marrow (in liver and spleen) and with other bone marrow diseases ***(Maedel and Sommer, 2002).***

Erythropoietin increases number of progenitor cells committed to erythropoiesis. One of the most impressive effects of EPO on erythroid cells, is its ability to maintain viability of these cells, irrespective of any effect on cycling and differentiation. Pattern of rapid DNA cleavage occurring in deprived erythroid cells of EPO, is characteristic of cells undergoing apoptosis (programmed cell death). So cell death is avoided in presence of EPO and erythroid cells are allowed to differentiate and form RBCs. Once restoring RBCs normal mass this ensues in a decrease of EPO level leading to rapid turn off of erythropoiesis allowing programmed cell death ***(Dessypris and Sawyer, 2009).***

Normal RBCs life span usually ranges from 90 to 120 days. So about 1% of RBCs are broken down every day and removed extravascular by macrophages of reticulo-endothelial system (RES). The main organ which removes old and damaged RBCs is the spleen, but also liver and bone marrow (BM) do ***(Dhaliwal et al., 2004).***

The microscopic examination of a peripheral blood smear provides a wealth of information to the clinician. Various forms of anemia may actually be diagnosed from abnormal red cell morphology reported on a blood smear examination. The report of abnormal white cell morphology may in fact indicate what additional testing may be required. Abnormal platelet morphology may detect a platelet function deficiency even when sufficient numbers of platelets have been reported from the analyzer ***(Jones, 2009).***

Preparation of blood films on slides

Blood films should be made on clean glass slides. Films made on cover glasses have negligible advantages and are unsuitable for modern laboratory practice. Films may be spread by hand or by means of an automated slide spreader, the latter being either a standalone instrument or a component of an automated blood cell counter ***(Bain and Lewis, 2011).***

For a reliable differential count on films spread on slides, the film must not be too thin and the tail of the film should be smooth (Figure 1). This should result in a film in which there is some overlap of the red cells, diminishing to separation near the tail, and in which the white cells in the body of the film are not too badly shrunken. Differences in

distribution of the various types of cells are probably always present to a small extent even in well made films, that's why we should spread the film rapidly by a smooth spreader at an acute angle ***(Briggs and Bain, 2011).***

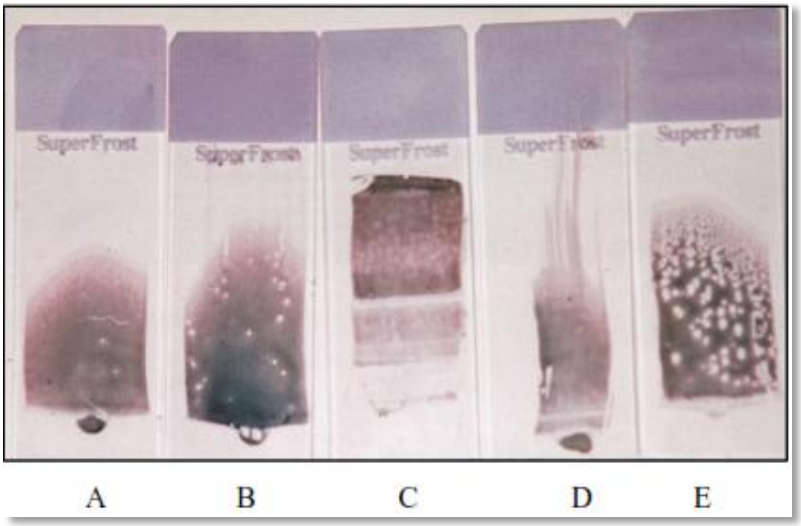

Figure (1): Blood films made on slides ***(Bain and Lewis, 2011).***

Staining of peripheral blood film

In practice staining with pure dyes are expensive, and it is sufficient to ensure that the stains contain at least 80% of the appropriate dye. Giemsa is the most complex dye. Leishman stain, which occupies an intermediate position, is still widely used in the routine staining of blood films. Leishman stain technique starts by drying the blood film then flooding the slide with the stain for 2 minutes. Diluting the stain with water for 5–7 min comes next. Finally washing it in a stream of buffered water until it has acquired a pinkish tinge (up to 2 min), wiping the back of the slide clean and setting it up right to dry ***(Bain, 2008).***

Examination of peripheral blood smear

Stained blood films should be examined in a systematic way. First the film should be examined without using the microscope, to make sure it is well spread (not too thick, too long or too short) and that its staining characteristics are normal. A film that is a deeper blue than other films stained in the same batch is usually indicative of an increase in the concentration of plasma proteins. This can be diagnostically important since it is often caused by multiple myeloma or by chronic inflammatory disease ***(Cheesbrough,2006).***

Next the film is examined microscopically at low power (e.g. with a 25x objective) so that a large part of the film can be scanned rapidly to detect any abnormal cells present in small numbers. Finally the film is examined at a higher power (e.g. with 40x or 50x objective) so that the detailed structure of cells can be assessed. The great majority of films can be evaluated perfectly adequately without using a 100x oil immersion objective. This can be reserved for making a detailed assessment of films that show significant abnormalities requiring further assessment ***(Bain, 2008).***

The examination of the blood smear should include evaluation of the red cell, white cell, and platelet

morphology. The red cell morphology evaluation should include examination for deviations in size, shape, distribution, concentration of hemoglobin, colors and appearance of inclusions ***(Jones, 2009).***

Red blood cell (RBC) morphology

1- Normal RBC morphology

Erythrocytes represent the most common cell type in adult blood. Human blood contains ~5×10^6 erythrocytes per microliter (normal range 4.7×10^6 to 6.1×10^6 for males and 4.2×10^6 to 5.4×10^6 for females); these cells have an average life span of 120 days. New erythrocytes are constantly produced in the bone marrow, which provides a niche consisting of endothelial cells of the vascular system, osteoblasts, stromal cells, hematopoietic cells, and the extracellular matrix. This complex niche supports direct cell–cell contact and exposure of the developing hematopoietic cells to cell adhesion molecules, growth factors, and cytokines ***(Kim et al., 2012).***

Erythroid cells at the terminal stages of differentiation have shed their nucleus, endoplasmic reticulum, and mitochondria, and, consequently, they are no longer able to proliferate. To maintain the red blood cell count in the ~5 L of blood of an adult individual, ~2.4×10^6 new erythrocytes have to be produced each second ***(Kim et al., 2012).***

The blood sample is examined under a microscope to assess the size, shape, and color of the red blood cells. Normal mature red cells can be described as round, elastic, non-nucleated, biconcave cells which have an area of central pallor that covers about one third of the cell. Normal mature red blood cells have an average diameter of 7.2 microns with a range of 6-9 microns, normal RBCs size appears to be the same as that of the nucleus of the small lymphocyte on the dried film ***(Kim et al., 2012).***

The normal red blood cell has a great excess of cell membrane for the quantity of material inside and hence deformability. RBCs lack a nucleus i.e. no DNA and no organelles and therefore cannot divide or replicate themselves like other cells. ***(Guyton and Hall, 2006).***

2- Red blood cell abnormalities

Normal and pathological red cells are subject to considerable distortion in the spreading of a film and it is imperative to scan films carefully to find an area where the red cells are least distorted before attempting to examine the cells in detail ***(Bain, 2011).***

The area of smear that is reviewed for morphologic abnormalities is of the most importance. The area to be reviewed should be in the thin portion of the smear where the red cells are slightly separated from one another or at

most, barely touching, with no overlap. The thin area should represent at least one third of the entire film. The reviewer should avoid the thicker portion of the slide where cells are overlapping and the edges of smear where cells may be distorted in size, shape, and color ***(Jones, 2009).***

A. Variation of red cell distribution

Two abnormalities of distribution may occur. When there is an increase in high molecular weight plasma proteins there is an effect on the electrical charge on the surface of the red cells and the cells sediment rapidly and form into stacks, a pile of coins. These stacks are referred to as rouleaux and the film is said to show increased rouleaux formation. The other abnormality of cell distribution is red cell agglutination. This is caused by an antibody against a red cell antigen ***(Bain, 2008).***

1. Rouleaux formation

Rouleaux formation is a phrase denoting the stacking of erythrocytes, generally in a curving pattern resembling a stack of coins ***(Gluckman et al., 2010).***

It is caused by an increase of asymmetric macromolecules such as globulin and fibrinogen. Associated clinical conditions include multiple myeloma, acute infection, inflammation and macroglobulinemia.

These alterations will result in an increased erythrocyte sedimentation rate (ESR) and a moderate to marked rouleaux in the peripheral blood film (PBF) ***(Constantino, 2014).***

The use of a saline dilution of the plasma disperses rouleaux. Peripheral smears reviewed in the thick portions of the smear and entire smears made too thick may appear to exhibit rouleaux. This is considered artifactual and should not be used until it is verified in the thin portion of the smear or a new slide is prepared ***(Jones, 2009).***

2. Red cell agglutination

Agglutination is an aggregation of red cells in to random clusters or masses. Agglutination is the result of an antigen antibody reaction within the body, and in cases of auto agglutination, the reaction is actually with the patient's own cells and the patient's serum or plasma. Such is the case with cold antibody syndromes, for example, cold hemagglutination disease and paroxysmal cold hemoglobinuria (PCH). Agglutination occurs at room temperature during sample preparation and appears as interspersed areas of clumping throughout the peripheral smear. The use of saline will not disperse these agglutinated areas; however, warming the sample to 37°C helps to break up the agglutinins, allowing for the possibility of normal slide preparation for morphology review ***(Jones, 2009).***

B. Variation in size (Anisocytosis)

The red cells may be abnormally small, i.e. microcytes, abnormally large i.e. macrocytes or they may show abnormal variation in size (anisocytosis). Anisocytosis is a feature of most anemias; when it is marked, both macrocytes and microcytes are usually present ***(Vajpayee et al., 2011).***

1. Microcytes

A microcyte is a small cell having a diameter of less than 7 µm and a mean corpuscular volume (MCV) of less than 80 fL**.** Anemia associated with microcytes are said to be microcytic. The hemoglobin content of these cells may be normal to decreased ***(Jones, 2009).***

The presence of microcytes usually results from a defect in haemoglobin formation. Microcytosis is characteristic of iron deficiency anemia, various types of thalassemia, and severe cases of anemia of chronic disease. Causes that are rarer include congenital and acquired sideroblastic anemias ***(Bain, 2011).***

2. Macrocytes

Macrocytes are cells that are approximately 9 µm or larger in diameter, having an MCV of greater than 100 fL. Anemias associated with these cells are referred to as macrocytic this could be classically found in megaloblastic anemia ***(Jones, 2009).***

C. Hemoglobin content (color variation)

Normal red cells are reddish brown with approximately the central third to quarter of the cell being paler. They are described as normochromic. Cells which have an area of central pallor more than a third of the diameter of the cell are said to be hypochromic and the film is said to show hypochromia. Cells which lack central pallor are said to be hyperchromic. Cells which show a greater than normal variation in the degree of hemoglobinization are said to show anisochromasia. Red cells which have a blue or lilac tinge are said to show polychromasia ***(Bain, 2008).***

1. Hypochromia

Any RBC having a central area of pallor of greater than 3 µm is said to be hypochromic. Most clinicians choose to assess hypochromia based on the mean corpuscular hemoglobin concentration (MCHC), which by definition measures hemoglobin content in a given volume of red cells (100 mL). When the MCHC is <32% the anemic process is described as being hypochromic and the slide reviewer should scan the peripheral smear for the presence of hypochromia. The most common condition manifesting hypochromia is iron deficiency anemia ***(Jones, 2009)***.

2. Hyperchromia

Red cells with a decreased surface to volume ratio and a decrease or absent central pallor may be described as hyperchromic. True hyperchromia exists when the MCHC is >36% and may be seen in the peripheral smears of patients with hemolytic anemias, including hemolysis caused by burns. Even though true hyperchromia does exist it is not reported as such. It is reported in terms of the cell abnormalities resulting from the increased volume of hemoglobin and the decreased surface area ***(Jones, 2009).***

3. Polychromasia

When RBCs are delivered to the peripheral circulation prematurely, their appearance in the Wright stained smear is distinctive. These red cells are described as polychromatophilic (diffusely basophilic) and are gray blue in color and usually larger than normal red cells. The basophilic color of the red cell is the result of the residual RNA involved in hemoglobin synthesis ***(Jones, 2009).***

Any clinical condition in which the marrow is stimulated, particularly RBC regeneration, will produce a polychromatophilic blood picture. This represents effective erythropoiesis as well as an assessment of bone marrow function. Examples of several conditions in which polychromasianoted include acute and chronic hemorrhage, hemolysis, and any regenerative red cell process ***(Jones, 2009).***

D. Variations in Shape (Poikilocytosis)