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A study on RBC histogram in different morphological types of anemia in comparison with peripheral blood smears in a tertiary care Centre in rural South India

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Abstract
Background: Erythrocytes are the most abundant and one of the most important cells in circulation in humans. They are biconcave, non-nucleated cells of the size of around 7.4 µm in diameter carrying hemoglobin. Various diseases and physiological conditions alters the morphology and contents of the red blood cells. The study of these alterations could be a major clue to the diagnosis and management of these diseases. Anemia is one of the most common morbidity associated with altered RBC morphology and content.
Aims & Objectives: The aims of this study were 1) To study RBC histogram patterns in different types of anemia (Microcytic hypochromic, Macrocytic Normocytic normochromic) 2. Comparison of automated RBC parameters in anemic patients with morphological features noticed on peripheral smear examination.
Methods: A Hospital based cross sectional study, in the Department of Pathology at Dr. SMCSI Medical College & Hospital, Karakonam, from November 2016-Novemeber 2018. Patients sent to hematology lab for evaluation of anemia were included in the study population with sample size calculated by the formula $1.962pq/d^2$.Blood samples of patients sent to hematology lab for evaluation of anemia by complete blood count (CBC) and peripheral smear examination.
Results: In this present study Of the total 488 anemia cases studied 286 were females, with a female to male ratio of 1.4:1.There were 121 females patients who belonged to the reproductive age group of 15-49 years. Maximum number of anemia cases (77.04%) was present in adult population of more than 15 years of age and the mean age group was 42 years.
Majority of the cases (64.58%) showed moderate anemia, 24.8% cases showed severe anemia and 10.62% cases showed mild anemia. Blood samples of 488 patients with anemia were evaluated along with the corresponding peripheral smear findings. The cases were Normocytic normochromic anemia, Microcytic hypochromic anemia, Dimorphic anemia and Macrocytic anemia as diagnosed by peripheral smear. In our study 64.2% of the cases were Normocytic normochromic anemia and 35.2% of cases were microcytic hypochromic anemia. Macrocytic anemia was observed only in 0.4% of cases and dimorphic anemia only in 0.2 % of the cases as diagnosed by peripheral smear examination.
Conclusions: RBC histogram is an automated analyzer generated graphical representation of RBCs with size being plotted on the X-axis and number being plotted along Y-axis. The study showed that subcategorization of histogram patterns did not generate any additional information from the histogram, than obtained from the broad categorization of Left shift curve, Right shift curve and Normal curve. This could probably be sample bias and hence require future studies. This study also showed that there could be overlapping of correlation of various histograms with different types of anemias diagnosed morphologically on the peripheral smear.

Keywords: Anemia, morphology, red blood cell, histogram, smear, hemoglobin

Introduction
Anemia is a major public health issue predominant in children and females of reproductive age group of low to middle-income population of developing countries like south Asia. Anemia constitutes an important diagnostic and clinical category of hematological disorders prevalent all over the world. As of 2010 statistics prevalence of anemia was approximately 32.9% worldwide resulting 68.36 million years lived with disability [1].
These changes can be evaluated either by an age tested laborious techniques, the peripheral blood smear evaluation. But a recent alternative is the use of automated hematology analyzer

which assesses all the cells passing through the predetermined chambers and gives a large number of blood cell indices. It works on basis of Coulter's principle. The analyzer used in this study was Sysmex XS 1000i.

Automated hematology analyzer counts all the non-nucleated cells in the range of 25-250 fL as RBCs and gives a corresponding histogram along with all the red blood cell indices. RBC histogram is an integral part of the automated hematology analysis with the size of cells being plotted along X-axis and number of cells being plotted along Y-axis. The normal histogram is a Gaussian curve. The histogram obtained from the study was compared with a reference curve and each histogram categorized was into a different type [2].

A study of RBC histogram done by Jithendra Chavda *et al* on 500 anemia cases along with peripheral smear examination, concluded that histogram could provide major hint on RBC disorder and guide in peripheral smear examination along with indices [3].

Another study by Sandhya. I & Muhasim, to assess the utility of RBC histogram along with peripheral smear revealed that histogram should be considered as a screening tool though not confirmatory for any pathological diagnosis [4].

Asuthosh S Kumar *et al* conducted a study on 60 anemia cases, with an objective of standardization and grading of abnormal blood cell morphology in peripheral blood smear, counter based red cell indices in case of anemia of various etiologies and comparative evaluation of peripheral blood smear examination and automated red cell indices including RDW, MCV, MCH, MCHC [5].

Peripheral smear is considered the standard technique for the morphological evaluation of red blood cells and histograms are always available as an integral part of the automated hematology analysis. So through this study we intended to evaluate the utility of histograms in narrowing down the differential diagnosis and hence arriving at a rapid diagnosis.

Anemia is a common health issue in developing countries especially among children and females of reproductive age group [7]. Complete blood cell count is a routine blood test performed in the clinical laboratory. It includes white blood cell count, hemoglobin, platelet count, and red blood cell indices [8]. Complete blood cell count is currently done using automated hematology analyzer and RBC histograms are an integral part of automated hematology analysis.

While majority of the clinicians and laboratory personnel are aware of the utility of RBC indices in the categorization of anemia, the significance of RBC histograms is being underestimated in the evaluation of anemia.

Automated hematology analyzer counts all the cells present in the sample passing through the predetermined chambers and hence the RBC histogram produced by the assessment of larger number of cells is likely to have a higher power of study.

Presence of multiple population of RBCs are more likely to be picked up by the histograms than the indices which are more of mathematical value. RBC histogram being a pictorical depiction of data can convey the message of the data easier even to a nonpathologist [4].

All these efforts to classify anemia are to detect easily rectifiable causes such as nutritional deficiencies, and anemias demanding urgent attention such as hemolysis, and anemia of renal insufficiency and bring them to attention of the physician or the pathologist. This helps them to narrow down their differential diagnosis and hence reduce the battery of tests required for the patient management [6].

In this study we attempt to classify histogram patterns into an increased number of subcategories and compare with the peripheral smear findings. The conventional method of categorization of histograms will also be compared with the peripheral smear. RBC indices will also be correlated with the corresponding peripheral smear findings. Thus we intend to make analysis of RBC histogram more user friendly and to help the clinician to narrow down the differentials even prior to peripheral smear confirmation by a patholologist. We also have analysed the pattern of anemia and its distribution among patients who have come to hospital, which may be a representation of the anemia pattern of the rural area.

Materials and Methods

Study Design: Hospital based cross sectional study.

Study Setting: Department of Pathology (hematology), Dr. SMCSI Medical College and Hospital, Karakonam.

Study Period: November 2016-November 2018

Sample Population: Patients sent to hematology lab for evaluation of anemia.

Study Sample: Blood samples of patients sent to hematology lab for evaluation of anemia by complete blood count (CBC) and peripheral smear examination.

Sample Size: 488, using the formula 1.962pq/d2

Where p is the proportion of normocytic anemia (17 %), 3
q = 100 –p = 83,
d is the allowable error 20 % of p = 3.4

Sampling Method: Consecutive samples of anemia diagnosed with a hemoglobin level lower than the normal limits for the age and sex of the individual.

Inclusion Criteria

1. Blood samples of all anemic patients presenting to Dr. SMCSI medical college, central laboratory with hemoglobin percentage below the lower limit of normal will be included in the study.
2. Patients of all age groups will be included in the study.

Exclusion Criteria

1. Incomplete request forms.
2. Samples for which both CBC and peripheral blood smears are not requested.

Tools and Technique

All the study samples were analyzed in the automated hematology analyzer SYSMEX-XS 1000i and a peripheral smear prepared.

Manual Technique for Peripheral SMEAR Preperation Using Leishman Stain: (49), (62)

- Draw blood from peripheral veininto vacutainer containing anticoagulant Ethylene diamine tetra-aceticacid (EDTA).

- Clean the glass slide.
- Place a drop of blood in the centre of the slideabout 1 cm from one end.
- Place a spreader,in front of the drop such that it makes an angle of about 30° with the slide. Move the spreader back so that it makes contact with the drop and the drop spread out quickly along the line of contact.
- Spread the drop of blood along the slide with steady rapid movement.
- The film should belabeled immediately using a pencil and allowed to dry.
- Pour undiluted Leishman stain on the dried slide with a dropper such that the stain covers the entire upper surface of the slide.
- Wait for 2 minutes.
- Pour buffer water on the slides, double the amount of stain used.
- Gently mix using a dropper.
- Wait for another 8-10 minutes till a metallic scum appears.
- Displace the stain by pouring running water from one side of the slide.
- Remove the slide from staining rack
- Keep the slide in slanting position to dry.
- Dip the slide in Xylene and mount in DPX
- Peripheral smear would be evaluated by two independent observers under microscope.
- Anemia would be morphologically categorized as Microcytic hypochromic anemia, Normocytic normochromic anemia, Macrocytic anemia, Dimorphic anemia.(33)
- Abnormal red blood cell morphology, immature cells and abnormal inclusions should be noted.

Operation of SYSMEX-XS 1000i

- Switch the power supply on by turning on the printer, IPU, Main Unit and other components.
- Execute the quality control analysis.
- Enter the patient details in the information processing unit of a calibrated analyzer.
- Select the manual mode.
- Mix the EDTA blood sample manually.
- Place the sample tube in the sample position in the sampler without removing the cap.
- Select the start option to initiate analysis.
- The flashing of green LED indicates sample aspiration, then the buzzer beeps to indicate the end of aspiration, and the LED goes out.
- The RBC indices and RBC histogram will be displayed on the screen of information processing unit.
- Operate the automated analyser and reagents as mentioned in the operator's manual. (63)

Operational Definition

Types of Histogram (3)

a. **Normal curve:** Curve which corresponding to reference histogram given by the machine. The peak of this curve roughly corresponds to 80 femtoliter.
b. **Left shift curve:** The curve which is shifted left with respect to the reference curve with peak and base being comparable to the reference curve.
c. **Right shift curve:** The curve which is shifted to right with respect to the reference curve with peak and base being comparable to the reference curve.
d. **Double peak:** Curve with two distinct peaks.
e. **Broad base:** When arithmetic width measured at the level of 20% of histogram i.e., RDW-SD is more than 50 fl.
f. **Normal curve with short peak:** Curve with peak at the same level of reference curve with respect to X-axis but less than half the height of reference peak.
g. **Normal curve with broad base:** Curve with peak at the same level of reference curve with respect to X-axis with a broad base.
h. **Left shift with short peak:** Curve which is shifted left with respect to reference curve and height of the peak less than half the reference peak.
i. Left shift with broad bas**e:** Curve which is shifted left with respect to reference curve and has a broad base.
j. **Right shift with short peak:** Curve which is shifted right with respect to reference curve and has a short peak.
k. **Right shift with broad base:** Curve which is shifted right with respect to reference and has a broad based.
l. **Normal' curve:** Broad category of histogram composed of Normal curve, Normal curve with short peak and Normal curve with broad base.
m. **Left shift' curve:** Broad category of histogram composed of Left shift curve, Left shift curve with short peak and Left shift curve with broad base.
n. **Right shift' curve:** Composed of only Right shift curve with broad base

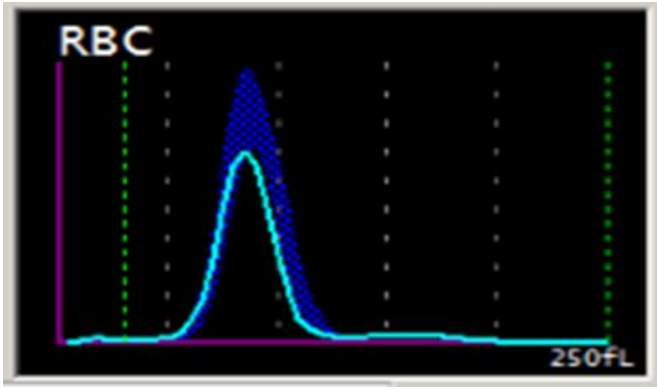

Fig 1: Normal Curve

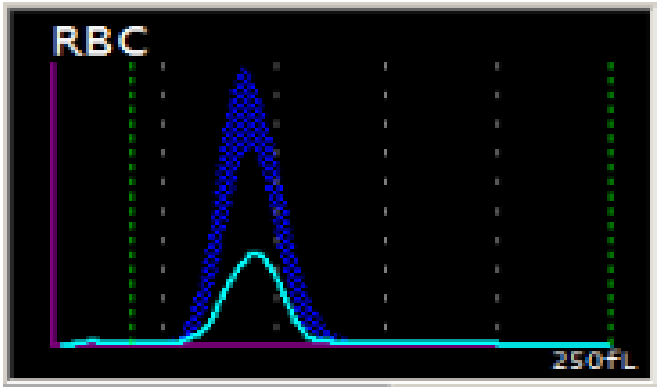

Fig 2: Normal curve with short peak

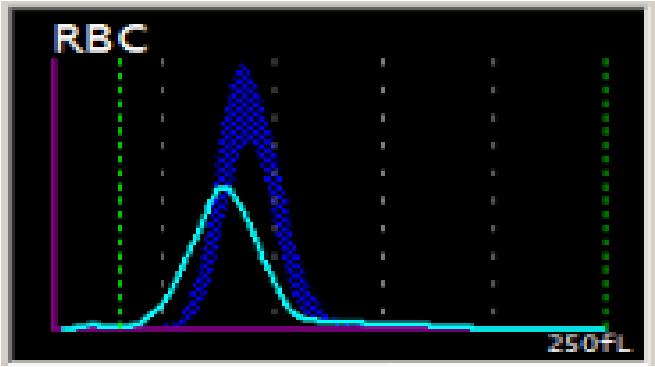

Fig 3: Normal curve with broad base

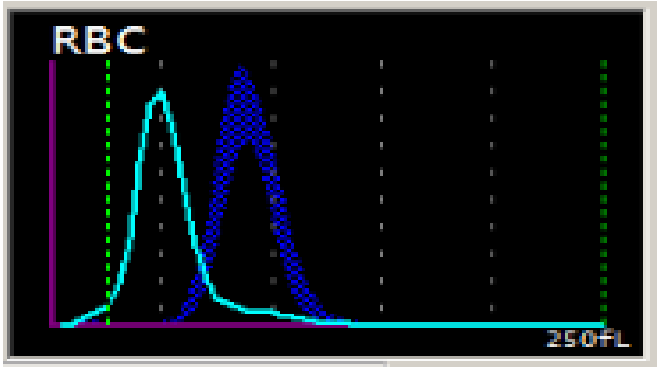

Fig 4: Left shift curve

Data Analysis

- Data was entered in Microsoft excel and analysis done with the help of IBM SPSS version 25 software.
- Proportion of various patterns of anemia was expressed as percentage with 95% confidence interval.
- Percentage was calculated to look for comparison between different types of anemia diagnosed peripheral smear examination.
- Pearson correlation coefficient was calculated to assess the correlation between RBC parameters and findings on peripheral smear.
- Confidence interval of 95% will be taken as statistical significance.

Results

Demographic Details of Study Population

Table 1: Gender Distribution of Anemia

Gender	Number	Percentage
Male	202	41
Female	286	59
Total	488	100

Of the total 488 anemia cases studied 286 were females, with a female to male ratio of 1.4:1.There were 121 females patients who belonged to the reproductive age group of 15-49 years.

The age distribution of anemia patients in the current study were as follows-

Table 2: Age Distribution of Anemia

Age	Frequency	Percentage %
Birth	2	0.410
One Month	2	0.410
2-6 Month	4	0.820
6 Months-6 Years	83	17.008
6-14 Years	21	4.303
>15 Years	376	77.049
Total	488	100

Maximum number of anemia cases (77.04%) was present in adult population of more than 15 years of age and the mean age group was 42 years.

Table 3: Grading Severity of Anemia

Severity of anemia	Frequency	Percentage
Mild	51	10.62
Moderate	310	64.58
Severe	119	24.8
Total	480	100

Majority of the cases (64.58%) showed moderate anemia, 24.8% cases showed severe anemia and 10.62% cases showed mild anemia.

Table 4: Pattern of anemia in the study population

Type of Anemia	Freqency	Percentage
Dimorphic Anemia	1	0.2
Macrocytic Anemia	2	0.4
Microcytic Hypochromic Anemia	172	35.2
Normocytic Normochromic Anemia	313	64.2
Total	488	100.0

In this study, blood samples of 488 patients with anemia were evaluated along with the corresponding peripheral smear findings. The cases were Normocytic normochromic anemia, Microcytic hypochromic anemia, Dimorphic anemia and Macrocytic anemia as diagnosed by peripheral smear. In our study 64.2% of the cases were Normocytic normochromic anemia and 35.2% of cases were microcytic hypochromic anemia. Macrocytic anemia was obsereved only in 0.4% of cases and dimorphic anemia only in 0.2 % of the cases as diagnosed by peripheral smear examination.

Table 5: Categorization of morphological type of anemia with respect to severity

Table 5A: Mild Anemia

Peripheral SMEAR	Mild Anemia	
	Frequency	Percentage
MHA	17	33.33
NoNc	33	64.70
Macrocytic	-	-
Dimorphic	1	1.9
Total	51	100

Table 5b: Moderate Anemia

Peripheral SMEAR	Moderate Anemia	
	Frequency	Percentage
MHA	83	26.77
NoNc	227	73.22
Macrocytic	-	-
Dimorphic	-	-
Total	310	100

Table 5c: Severe Anemia

Peripheral Smear	Severe Anemia	
	Frequency	Percentage
MHA	70	58.8
NoNc	47	39.5
Macrocytic	2	1.7
Dimorphic	-	-
Total	119	100

Majority of the subjects of Normocytic normochromic anemia had (227 cases) moderate degree of anemia and 47 had severe anemia followed by mild anemia seen in 33subjects.Of the 172 Microcytic hypochromic anemia cases majority i.e.83 cases had moderate degree of anemia 17 had mild anemia. Both the 2 cases of Macrocytic anemia were severe in grade and the single case of Dimorphic anemia was mild in degree. This categorization did not include children less than 6 months of age.

Table 6: Histogram Patterns (95% C.I)

Type of histogram	Frequency	Percentage
Left Shift	161	33
Normal	126	25.8
Normal (Short Peak)	113	23.1
Left Shift (Broad Base)	38	7.8
Left Shift (Short Peak)	34	7
Normal (Broad Base)	7	1.4
Right Shift (Short Peak)	5	1
Double Peak	4	0.8
Total	488	100

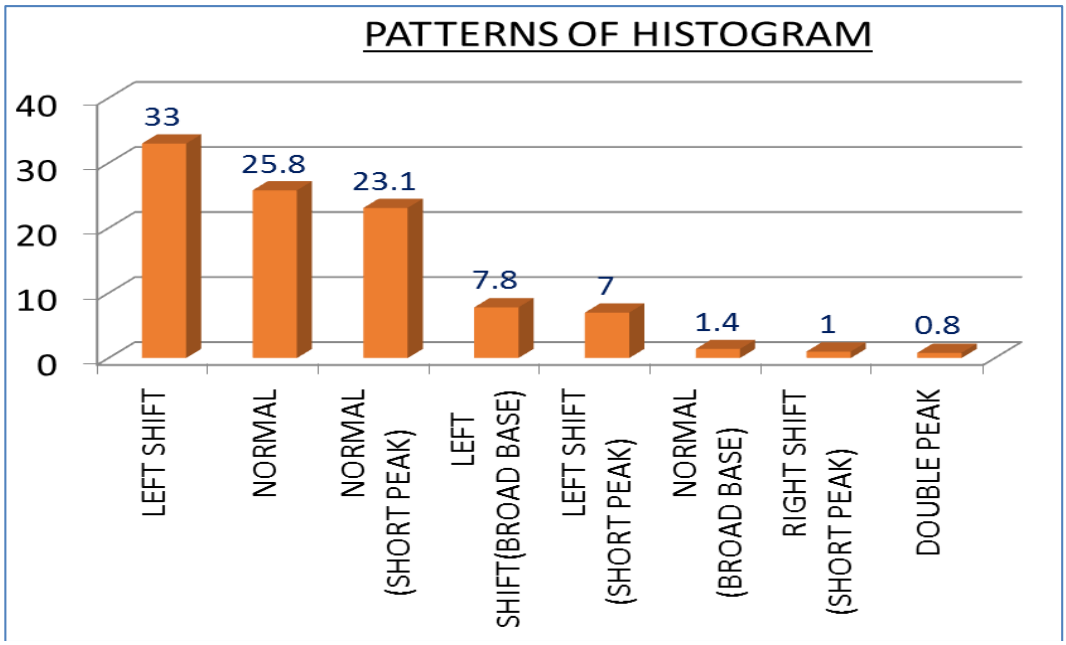

Fig 5: Distribution of Histogram Patterns

Of a total of 488 cases studied, majority of the histograms showed left shift curve in 161 (33%) cases, followed by normal curve in 126 (25.8%) cases.Other histograms patterns observed less frequently were Normal short peak curve in 113 (23.1%) cases, Left shift curve with broad base in 38 (7.8%) cases, Left shift curve with short peak in 34 (7%) cases, Normal curve with broad base in 7 (1.4%) cases, Right shift curve with short peak in 5 (1%) cases and bimodal curve in 4 (0.8%) cases.

Conclusion

RBC histogram is an automated analyzer generated graphical representation of RBCs with size being plotted on the X-axis and number being plotted along Y-axis. The study showed that sub categorization of histogram patterns did not generate any additional information from the histogram, than obtained from the broad categorization of Left shift curve, Right shift curve and Normal curve. This could probably be sample bias and hence require future studies. This study also showed that there could be overlapping of correlation of various histograms with different types of anemias diagnosed morphologically on the peripheral smear. This could be due to causes that may not be picked up by the examination of peripheral smear alone, like presence of a small number of fragmented RBCs may give the impression of Normocytic normochromic anemia on peripheral smear but the histogram may give left shift curve. The study also showed that a short peak of histogram is suggestive of a reduced RBC population. Hence, clues given by the RBC histogram should be thoroughly analyzed by the pathologist prior to the evaluation of peripheral smear examination.

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