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Utility of automated cell counter histograms in reporting peripheral smears in anemia

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Abstract
Background: Over the past few years complete blood count (CBC) by the automated hematology analyzers and microscopic examination of peripheral smear have complemented each other to provide a comprehensive report on patient's blood sample. Many times it is seen that histogram patterns show varying features when a simultaneous peripheral smear is reported. It is also seen that there are many limitations when manual peripheral smears reporting is done for example: peripheral smear reports are subjective, labor intensive and statistically unreliable. However microscopic peripheral smear examination also have their advantages.
Aims and Objectives: This study was conducted to create a guide for the laboratory personnel and clinicians with sufficient accuracy to presumptively diagnose morphological classes of anemia directly from the automated hematology cell counter forms and correlate with morphological features of peripheral smear examination.
Materials and Methods: The present study was undertaken in the central diagnostic laboratory at the AJIMS. 500 patients with anemia were included. 3 ml of EDTA blood sample was collected and a histogram was obtained after thorough mixing. The automated analyzer used in this hospital LABLIFE D5 SUPREME i.e. 5 part differential automated analyzer was used for the study. A simultaneous peripheral smear was also prepared according to standard operating procedures and stained by leishman stain. This peripheral smear was reported by the pathologist.
Results: In the present study we noted that microcytic hypochromic anemia was the most common (72.2%) anemia. The histogram patterns correlated with the peripheral smear findings in majority cases of normocytic normochromic anemia, microcytic hypochromic anemia and macrocytic hypochromic anemia.67% histogram pattern showed left shift. 19.4% histograms showed normal curve, 11.8% showed broad base curve and 0.8% graphs showed right shift and bimodal curve. Dimorphic anemia cases showed varied patterns of histograms and required thorough examination by peripheral smears.
Conclusion: Peripheral Smear examination is a sensitive and a gold standard when it comes to diagnosing red blood cell disorders. Red cell histogram patterns and the red cell indices obtained from the hematology automated analysers act as an adjunct to the visual examination of peripheral smears.

Keywords: Automated analyzer, peripheral smear, red cell indices

1. Introduction
The peripheral blood smear has been the main diagnostic aid in establishing the etiology of an anemia. Examining the blood films routinely has facilitated interpretation of various hematological disorders. Thirty to forty years ago, laboratory hematology was labor intensive and time consuming. Procedures were manual. Reagents were prepared in the laboratory from raw chemicals. Hemoglobin measurement was based on the cyanmethemoglobin method, which involved tedious procedure. The automated hematology analyzer has replaced the traditional manual methods for hematological parameters as the initial screening and detection system for hematological abnormalities in modern clinical setups. From the earlier instruments that used electrical impedance as the sole counting principle for blood cells, modern day analyzers, in addition, use conductivity differences, cytochemical staining, light scatter, and flow cytometric principles. While enhancing the speed, accuracy and precision of test results, this has also added a new dimension to hematology reporting. However, even in the wake of much technological advancement, the attention to numerical data with regards to the interpretation of test results has not changed. In most laboratory setups, traditional emphasis has been placed on verifying the automated data, an exercise that has outlived its importance [1].

As the automated analyzers become more advanced, their precision has shown enormous improvement and manual blood smear review rates have been on a steady decline [2]. There is a need for hematologist to give more clinically useful opinions on blood samples run on automated analyzers instead of signing out an automated report. Automated complete blood count and differential counts has reduced the number of technologists needed for performance of these tests.

The RBC histogram is an integral part of automated hematology analysis and is now routinely available on all automated cell counters. The histograms provide major clues in diagnosis and management of significant red cell disorders. The overall pattern of histogram by itself in the red cell distribution curve is meaningless unless it is compared with a reference normal curve and confirmed microscopically. Unfortunately technologists may have a limited understanding in correlating the graphic displays with the morphological findings [3]. This is probably because graphical representation of results such as scatter plots or histograms have been largely ignored in favor of the RDW, hemoglobin distribution width, and reticulocyte hemoglobin content that provide very useful information along with the red cell indices that have been traditionally used [4, 5, 6].

Despite the sophistication of present day instruments, there is still need to depend on manual techniques for primary calibration. This highlights the importance of maintaining the manual technical skills, and to ensure this by appropriate technician training program, despite the temptation to leave it all to the machines. This present study was designed and conducted to determine the relationship between Lablife D5 supreme automated hematology analyzer blood counts and manual counts using human subject's blood samples at the department of pathology, AJIMS.

2. Aims and objective

The present study is done with the following aims and objectives:

1. Interpretation of histograms in normal persons and patients with different types of anemia
2. Comparison of automated histogram patterns with morphological features noticed on peripheral smear examination.

3. Materials and Methods

Source of data

The present study was undertaken in the central diagnostic laboratory at the AJIMS, Mangalore. A total of 500 patients with anemia were studied.

Method of collection

3ml of EDTA venous blood sample was collected from the patient and a histogram was obtained after thorough mixing of the sample. The automated analyzer LABLIFE D5 SUPREME i.e. 5 part differential automated analyzers was used for the study. Simultaneously a peripheral smear was prepared according to standard operating procedures and stained by Leishman stain. This peripheral smear was reported by pathologist who was not privy to histogram during the reporting of peripheral smear.

Inclusion criteria

1) All anemic patients with hemoglobin percentage less than 11.5gm% were included
2) Patients of all age groups were included in the study

Exclusion criteria

1. All cases of anemia that have undergone blood transfusion will be excluded from the study.
2. Inadequate quantity of blood sample for automated analyzer (< 3ml) will be excluded

Staining of thin blood films

For the current study Leishmans stain was used due to easy feasibility.

Preparation: Dissolve 0.2g of powered Leishmans dye in 100ml of acetone-free methyl alcohol in a conical flask. Warm it to 50º C for half an hour with occasional shaking. Cool it and filter it.

Procedure: Pour Leishmans stain drop wise on the slide and wait for 2 minutes. This allows fixation of blood film in methyl alcohol. Add double the quantity of buffered water over the slide. Mix by rocking for 8 min. Wash in water for 1 to 2 minutes. Dry in air and examine under oil immersion lens of the microscope.

Statistical data analysis

A qualitative analysis of the data was done using Pearsons Chi square test and Fisher exact test wherever appropriate.

4. Results and analysis

Age and Gender Wise Distribution of Study Subjects

Majority of females (58.8%) were in reproductive age group.

After 40 yrs of age males (48.26%) were seen to be affected more than females (30.37%).

Table 1: Age and gender wise distribution

Age(Years)	Sex		Total No (%)
	Male-No (%)	Female-No (%)	
1-10 Years	37(56.1%)	29(43.9%)	66(100%)
11-20 Years	23(40.4%)	34(59.6%)	57(100%)
21-30 Years	32(29.1%)	78(70.9%)	110(100%)
31- 40 Years	28(37.3%)	47(62.7%)	75(100%)
41- 50 Years	39(54.9%)	32(45.1%)	71(100%)
51- 60 Years	37(74%)	13(26.0%)	50(100%)
61-70 Years	21(43.8%)	27(56.3%)	48(100%)
Above 71 Years	14(58.3%)	10(41.7%)	23(100%)
Total	231(46.1%)	270(53.9%)	500(100%)

Classification Of Anemia Based On Heamoglobin Values According to the hemoglobin values anemia was divided into following categories: Mild (Hb<11gms), Moderate (Hb 7-10 gm%) and Severe (Hb<7gm%).
Out of the 500 subjects' majority cases (50.3%) showed moderate anemia.

Table 2: Hemoglobin wise distribution

Hemoglobin (gm %)	Females-count (%	Males-count (%)	Total-count (%)
Mild	96(35.6%)	80(34.6%)	62(12.4%)
Moderate	133(49.3%)	119(51.5%)	252(50.3%)
Severe	33(12.2%)	29(12.6%)	176(35.1%)

Cases of Anemia Based on Peripheral Blood Smear Examination.

Table 3: Peripheral Smear findings

Peripheral Smear Findings	Frequency (Percentage)
Normocytic Normochromic	70(14.0%)
Microcytic Hypochromic	361(72.2%)
Macrocytic Anemia	2(0.4%)
Dimorphic Anemia	67(13.4%)
Total	500(100%)

Cases of Anemia Based On Peripheral Blood Smear Examination.
Morphologically we adhered to strict criteria to diagnose anemia.
Microcytic Hypochromic anemia had predominatly microcytic hypochromic red blood cells.
Macrocytic anemia had predominantly macrocytic red blood cells with or without a minor fraction of normocytic normochromic red blood cells.
Normocytic normochromic anemia had normocytic normochromic cell population with or without a minor fraction of microcytes or macrocytes.
Dimorphic anemia were diagnosed based on arbitrary categorization with multiple populations of cell i.e Microcytic hypochromic with macrocytes and normocytes.

Cases of anemia based on histogram pattern
Out of total 500 cases
395 (67%) cases showed Left Shift.
19.4% showed Normal Curve,
0.8% showed Bimodal Curve
0.8% showed Right Shift and
11.8% showing Broad Base curve.
[Refer figures 18, 20, 22, 24 & 26 for histogram patterns]

Table 4: Histogram Pattern

Histogram pattern	Frequency(Percentage)
Normal curve	97(19.4%)
Left shift	395(67.0%)
Right shift	4(0.8%)
Bimodal curve	4(0.8%)
Broad base	60(11.8%)
TOTAL	500(100%)

Correlation between Peripheral Smear Findings and Histogram Pattern.
In the table below we can see that most of the findings on peripheral smears can be correlated with the histogram patterns.

Table 5: Comparison between peripheral smear findings and histogram pattern

Peripheral Smear	Normal curve	Left shift	Right shift	Broad base	Bimodal curve	Total
Normocytic Normochromic	46	22	1	1	0	70
Microcytic Hypochromic	43	293	0	25	0	361
Macrocytic Anemia	0	0	2	0	0	2
Dimorphic Anemia	6	26	0	31	4	67
Total	95	341	2	58	4	500

Table 6: Correlation of Peripheral Smear findings and Histogram patterns

	N.N Count (percent)	M.H Count (percent)	M.A Count (percent)	D.A Count (percent)	Total
PSmear findings	70(14.2)	354(71.8)	2(0.4)	67(13.6)	493(100)
Histogram Pattern	97(22.1)	334(76.1)	4(0.9)	4(0.9)	439(100)
Total	167(17.9)	688(73.8)	6(6)	71(7.6)	932(100)

P<0.001 very highly significant, Fishers exact test =68.62
On applying Fishers exact test to the two variables we got the P <0.001 which indicated that there is very high significant difference when we compare the anemia diagnosed by histograms and by manual examination of peripheral smear.

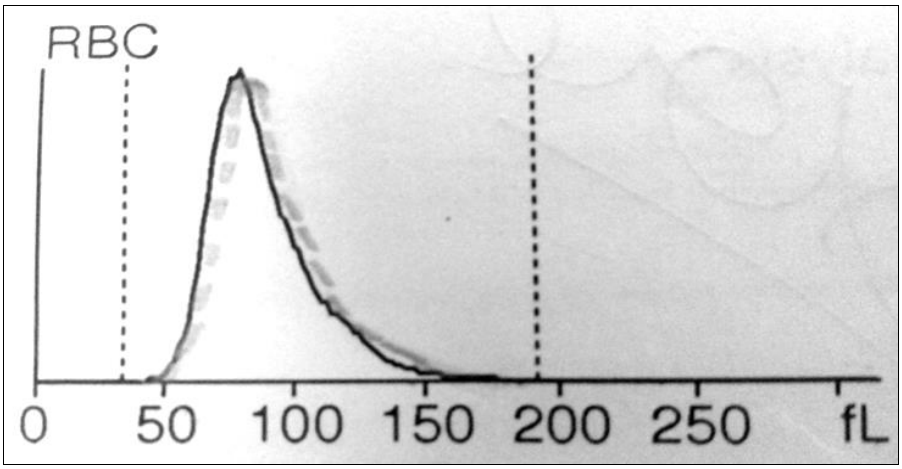


Fig 1: Normal curve seen in Normocytic normochromic Anemia

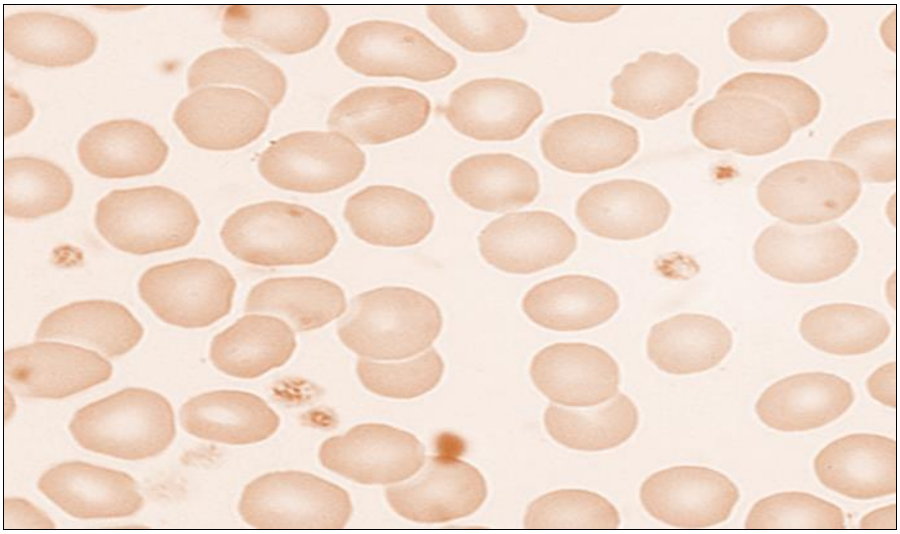


Fig 2: Peripheral Blood Smear In Normocytic Normochromic Anemia

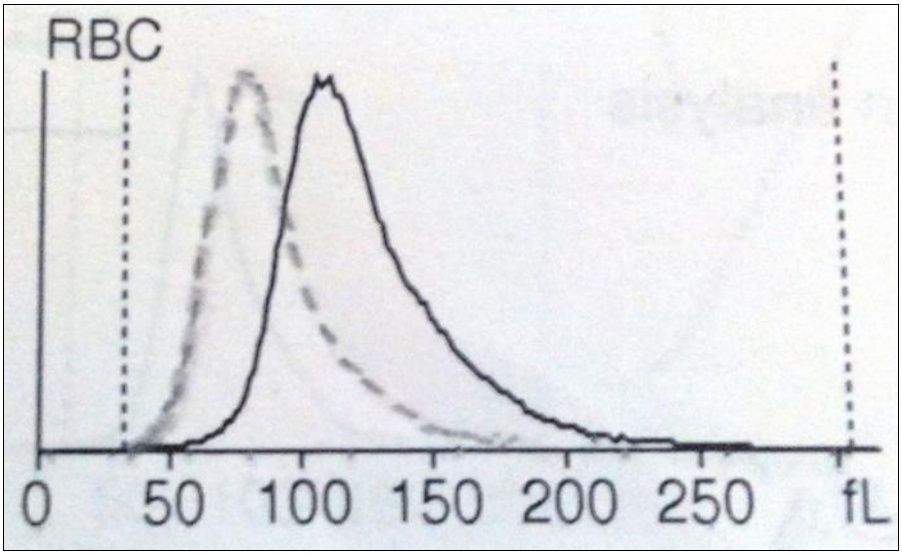


Fig 3: Histogram showing right shift in macrocytic anemia

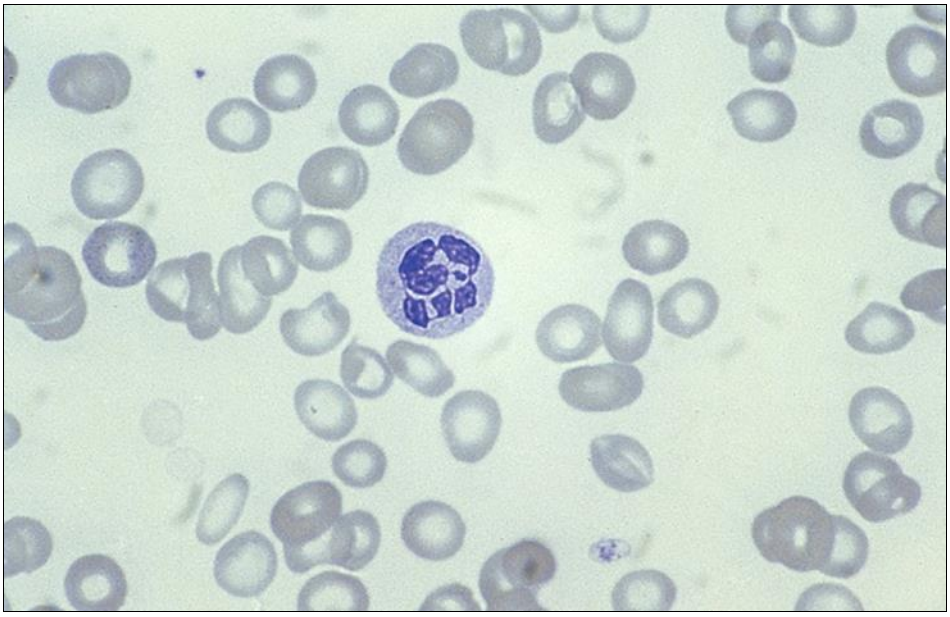


Fig 4: Peripheral Blood Smear in Macrocytic Anemia

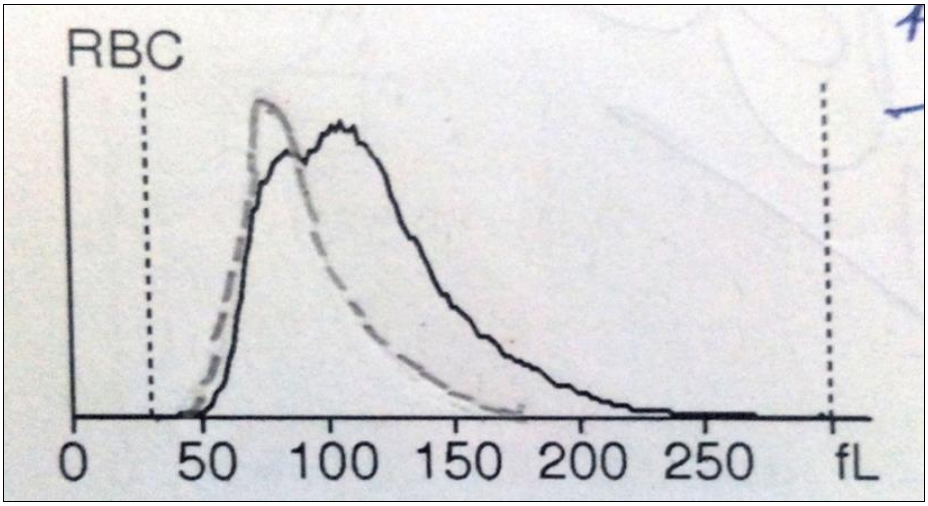


Fig 5: Bimodal histogram pattern in dimorphic anemia

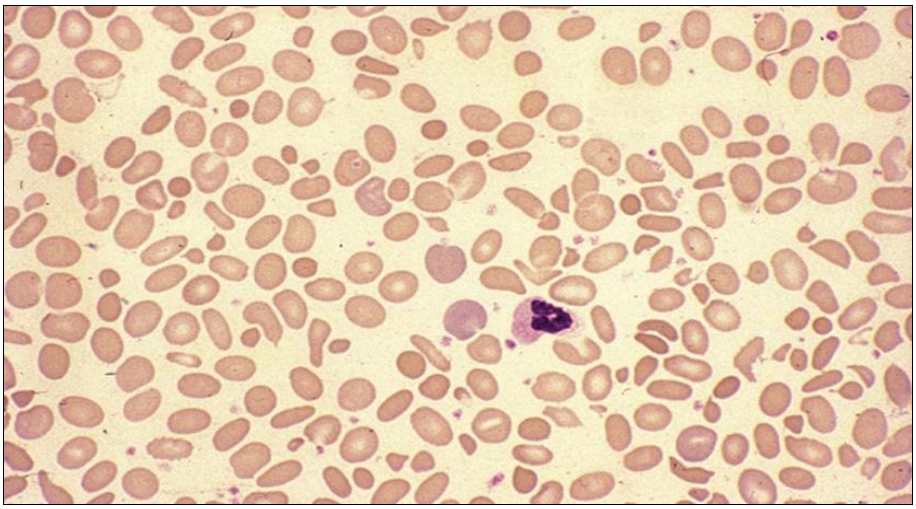


Fig 6: Peripheral smear in Dimorphic Anemia

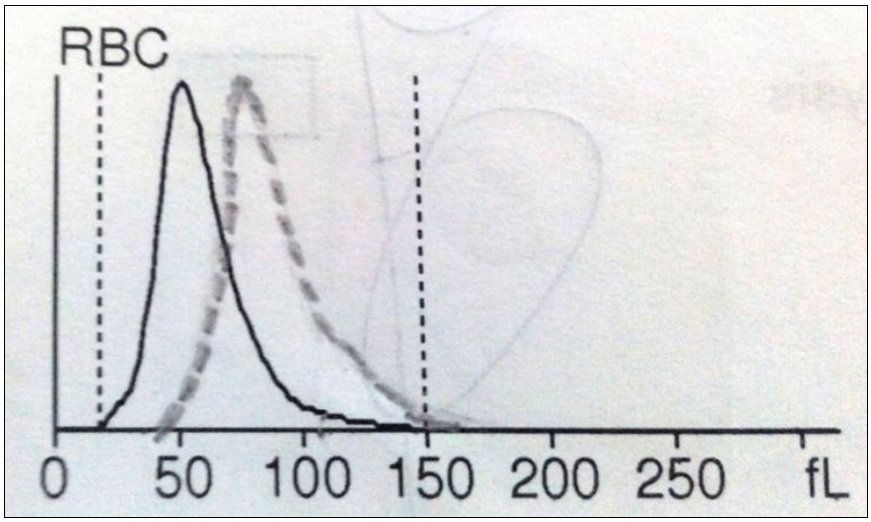


Fig 7: Left shift of histogram in microcytosis

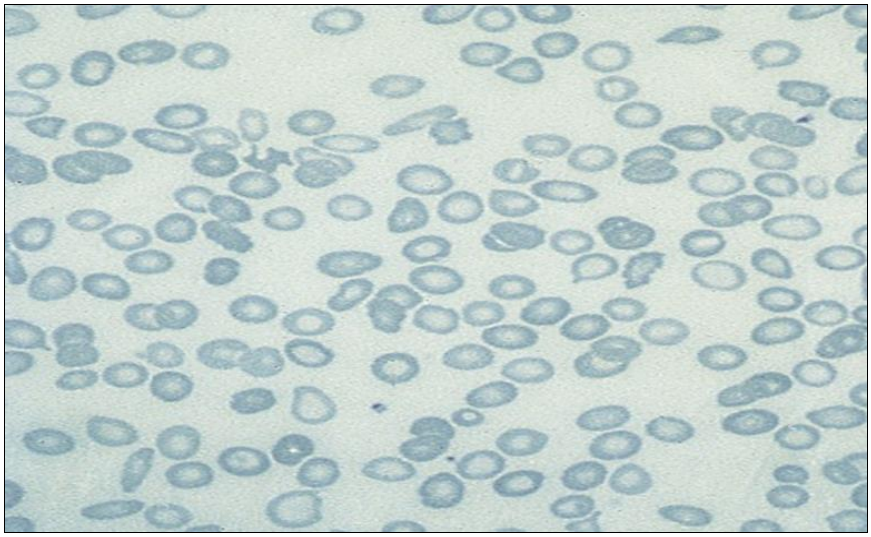


Fig 8: Peripheral smear in microcytic hypochromic anemia

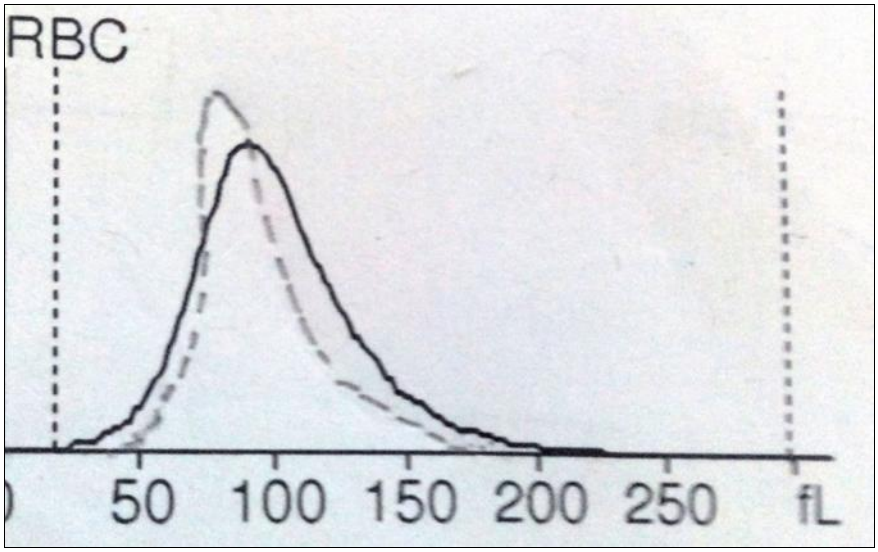


Fig 9: Broad base curve in dimorphic anemia

6. Discussion

Age

Our study showed a predominance of age group between 21-49 years, the mean being 36.08 years. This can be compared to other studies shown in the table below.

Table 7: Comparison of mean age group of anemia patients

Age (yrs)	Japheth E Mukaya *et al.* (2009) [7]	Kumar *et al.* (2013) [8]	Present study (2014)
Mean	40.7	20.47	36.08
Median	37	15	34

Table 8: Comparison patients in different age groups

Age Group	Japheth E *et al.* (2009) [7]	Present study (2014)
<30yrs	26.3%	46.3%
31-40yrs	32.5%	14.9%
41-50yrs	18.5%	14.1%
51-60yrs	9.8%	9.98%
>60yrs	12.9%	14.37%

Sex distribution

The total number of cases of Anemia in our study was 500. Out of these 500 majority of the cases were females (53.9%) compared to males (46.1%). This is comparable to the previous studies done as shown in table below.

Table 9: Comparison of Sex Distribution

	Cook *et al* (1976) [9]	Japheth E Mukaya *et al.* [7] (2009)	Present study (2014)
Females	55.69%	60%	53.9%
Males	44.24%	40%	46.1%

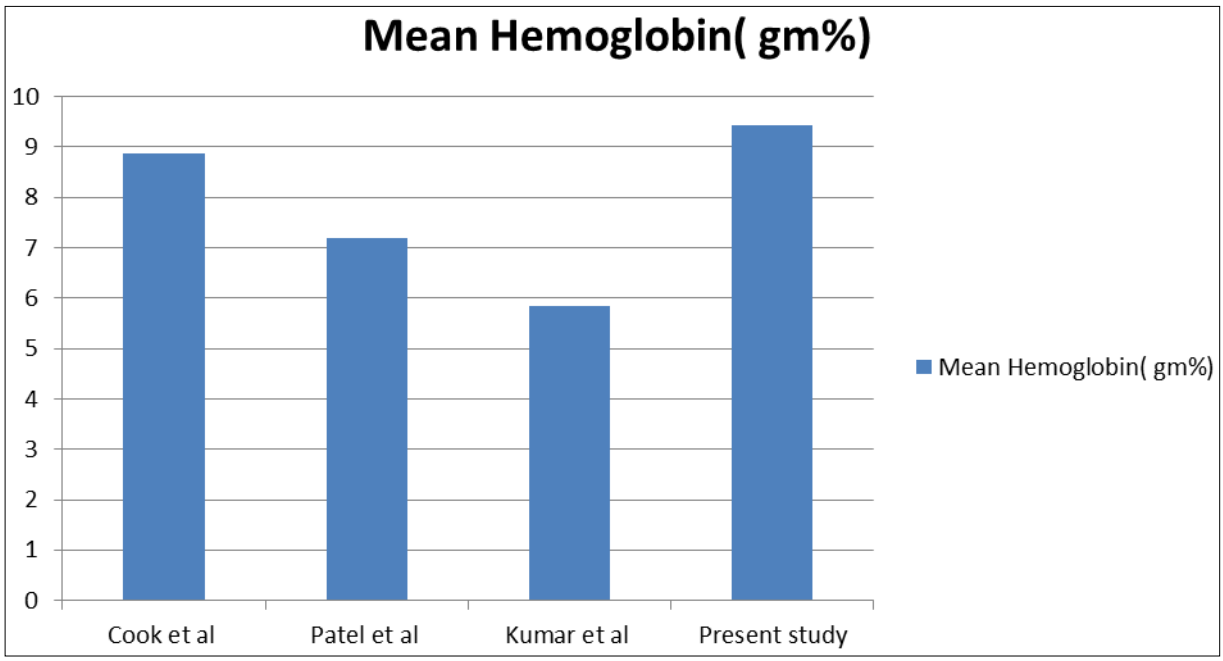
In our study it was seen that out of 500 cases majority of cases fall in the adult age group of 21 to 49 years and among 270 female patients majority (70.8%) fall in reproductive age group. This results were in concordance with the studies conducted by Kumar *et al*, Cook *et al* and Japheth *et al*. This can be explained as the period of adolescence and adult group is a period of intense growth and development and iron is in high demand as it is present in all body cells and is fundamental for basic physiological processes such as Hemoglobin formation. The body needs more iron when it grows rapidly and when frequent blood loss occurs (eg. Menstruation) thus women in reproductive age group are at high risk of developing iron deficiency anemia. After the age of 40 yrs males were seen to more affected than females.

Mean hemoglobin

In our study we considered all cases with hemoglobin less than or equal to 11gms%. The mean hemoglobin was 9.42gm%. Majority 50% of cases had hemoglobin 7-10gm/dl. This was compared with other studies as shown in table below:-

Table 10: Comparison of mean Hb value

Hemoglobin level	Cook *et al.* (1976) [9]	Patel *et al.* (2009) [10]	Kumar *et al.* (2013) [8]	Present study (2014)
Mean	8.87gm%	7.2gm%	5.85gm%	9.42gm%



Graph 4: Comparision of mean Hb value

Peripheral blood smear examination

Out of total 500 cases, majority (70.7%) cases showed Microcytic Hypochromic blood picture. This was compared to other studies as shown in table below.

Table 11: Comparision of cases of anemia based on peripheral blood smear examination

Peripheral smear findings	Patel *et al.* (2009) [10]	Japheth *et al.* (2009) [7]	Kumar *et al.* (2013) [8]	Yoginder *et al.* (2014) [11]	Present study (2014)
Normocytic normochromic anemia	24%	31%	31.7%	-	14%
Microcytic Hypochromic Anemia	72%	54.5%	33.3%	66.8%	72.2%
Macrocytic Anemia	4%	10%	21.7%	22.2%	0.4%
Dimorphic Anemia	-	8.6%	13.3%	11%	13.4%

The above comparision shows that, most common morphological type of anemia was Microcytic Hypochromic anemia (72.2%) followed by Normocytic normochromic anemia. Iron defiency anemia is the most common cause of microcytic hypochromic blood picture. WHO has estimated that prevalence of anemia in pregnant women is 14 per cent in developed and 51 per cent in developing countries and 65-75 percent in India. About one third of the global population (Over 2 billion) are anaemic [12]. Prevalence of anemia in all the groups is higher in India as compared to other developing countries [13].

Comparison between anemia diagnosed on peripheral smears and automated cell counter histograms

Very few studies have been conducted on the utility of red cell histograms in identifying common haematological disorders [14]. With most of the studies favouring white cell histograms and their use in identifying and characterizing leukemia blast populations [15].

The RBC histogram is an integral part of automated hematology analysis and is available routinely on all automated cell counters. The histogram in association with other CBC parameters such as RBC distribution width and mean corpuscular volume has been found abnormal in various haematological conditions [14, 16, 17, 18].

In the present study 500 patients of anemia were analysed and we compared their peripheral smear report with the Red blood cell histogram pattern obtained from LAB Life D5 supreme i.e 5 part differential automated analyzer. In view of maximizing the usefulness of the histogram a dotted line depicting a refrence normal curve was drawn super imposed on every red cell histogram so any discernible deviation from that curve can be clearly delineated for contrast. We noticed that in smears reported as microcytic hypochromic anemia 81.1% histograms showed left shift, 65% of nomocytic normochromic smears showed normal curve and all the smears having macrocytic blood picture showed right shift pattern of histogram. Thus we can see that histogram are useful diagnostic aid when it comes to normocytic normochromic anemia, microcytic hypochromic anemia and macrocytic anemia.

However the dimorphic anemia showed different histogram patterns from simple curve to complex curves. In the smears reported as dimorphic anemia we noticed that only 5.9% of histograms showed bimodal curve, whereas majority 46.26% showed broad base histogram pattern and 38.8% showed left shift histogram curve. The broad base curve can be explained by the presence of multiple populations of cells of varying sizes (i.e. normocytic, microcytic and macrocytic). Our study was in concordance with the study conducted by Constantino *et al*. in 2010.

Using Fisher Exact test and comparing the two variables i.e peripheral blood smear reports with histogram patterns the p values showed very high significant difference between the two variables. This difference was largely due to dimorphic anemia cases which was in concordance with Constantino *et al*. [19]

The bimodal red cell histograms are usually associated with therapeutic transfusions and / or hematinic agent response to microcytic and macrocytic anemia, but they may also indicate other haematological disorders as shown in following table.

Table 12: Conditions associated with dimorphic red cells [20, 21, 22-27]

1.	Early iron developing microcytic population
2.	Folate/vitamin B12 developing macrocytic population
3.	Post-iron treatment of iron deficiency anemia
4.	Post-iron treatment of iron deficiency with megaloblastic anemia
5.	Post-iron treatment of megaloblastic anemia
6.	Post-iron treatment of megaloblastic anemia with iron deficiency
7.	Post-iron transfusion macrocytic anemia
8.	Post-iron transfusion microcytic anemia
9.	Iron deficiency anemia with either folate or vitamin B12 deficiency
10.	Sideroblastic anemia (Myelodysplasia)
11.	Hemolytic anemia (Reticulocytosis, spherocytosis, fragmentation, pyropoikilocytosis)
12.	Cold/warm auto agglutination
13.	Erythropoietin-induced erythropoiesis
14.	Delayed transfusion reaction
15.	Homozygous hemoglobinopathies (Admixture of many RBC forms)
16.	Myelofibrosis (Admixture of extramedullary hematopoiesis)

In Dimorphic anemia the histogram pattern, the centeredness and the width shows the variations in the RBCs. The dimorphic blood picture will look like a dual population of microcytic and normocytic or normocytic and macrocytic red cells or a admixture of small, normal and large cells of different sizes and forms with or without normal red blood cell indices which can mislead the diagnosis if we rely on automated values alone, thus it is important to examine the peripheral blood smear to examine all the populations of the cell. Practically since dimorphic is usually associated with abnormal red cell populations, morphological findings should be correlated with the graphical and numerical data for better interpretation of results.

Naveen *et al*. in 2013 showed similar findings in dual population of Red blood cells. He stated that dual RBC population can be identified with most dramatic representation on histograms.

7. Summary

1. A total of 500 patients blood samples were processed during the study period
2. All cases had anemia with hemoglobin less than 11gm/dl.

3. The cases consisted of Normocytic normochromic anemia (14%), Microcytic hypochromic anemia (72.2%), Macrocytic anemia (0.4%) & Dimorphic anemia (13.4%).
4. The maximum number of patients there in between 21-49 yrs. mean age being 36.08 years.
5. Out of 500 patients 46.1% were male and 53.9 % were female.
6. Anemia graded to Mild (above 9.0g/dl), Moderate (6-9g/dl) and Severe (below 6g/dl). Out of 500 cases 35.1% were mild anemia, 12.4 % were severe anemia and 50.3% with moderate anemia.
7. Out of 500 cases 19.4% were normal histogram, 67% were left shift histogram, 0.8 were right shift histogram, 0.8% were bimodal curve and 11.8% broad basecurve. Majority of the histogram were left shift histogram (67%).
8. In cases reported as normocytic normochromic anemia on peripheral smears 65% showed normal curve. In cases reported as microcytic hypochromic anemia 81.8% showed left shift. In cases of macrocytic anemia 100% showed right shift. The peripheral smears reported as dimorphic anemia 5.9% showed bimodal curve 38.8% showed left shift. Majority of the curves in dimorphic anemia showed broad based curve (46.26%).
9. The histogram pattern correlated with majority cases of microcytic hypochromic, normocytic normochromic and macrocytic anemia. However variations of histogram patterns were seen in dimorphic anemia.

8. Conclusion

Red cell histogram from automated hematology analyzer provides valuable information regarding the hematological conditions. Very few studies have concentrated on RBC histograms while giving more importance to WBC histogram and leukemic blast populations. Our study showed a significant correlation between RBC histograms and peripheral smear diagnosis in Microcytic Hypochromic, Normocytic Normochromic and Macrocytic Anemia. However the correlation between histogram patterns and peripheral smear diagnosis in dimorphic anemia posed queries regarding the authenticity of histocytograms. Hence it was concluded that in the age of molecular analysis and automation, peripheral smear examination along with clinical history is an important diagnostic tool while treating patients. Red cell histograms along with numeral parameters like MCV, MCH, MCHC and RDW act as an adjunct to visual examination of peripheral smears.

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