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Comparison between Morphological Aided Cytochemical Stains (Sudan Black B, PAS) and Flowcytometry in Diagnosis and Classification of Acute Leukemia's.

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ABSTRACT

The diagnosis of AL undergoes a stepwise approach. First the distinction of an acute leukemia from other hematological diseases . Second is differentiating AML and ALL . The third is the classification of AML and ALL into categories that define treatment and prognosis. The Aim is to assess the importance of morphologic and cytochemical diagnosis in comparison with flowcytometric diagnosis in acute leukemia. A total of 79 bone marrow specimens were diagnosed as acute leukemia based on morphology, cytochemistry and by flowcytometry. The results based on morphology are 58.21% diagnosed as AML, 37.9 % diagnosed as ALL and 3.79% as AUL. In AML, SBB was positive in 78.2 % of cases ,sub classified from M1 to M4, while 21.7% had negative SBB subclassified as M5a, M5b and M6. PAS were negative in all AML cases. In ALL, PAS was positive in 60% of cases and SBB was negative in all cases. AUL cases had negative results in both stains. Flowcytometric results are: AML; M0 (2.53%), M1(2.53%), M2 (24.05%), M3 (5.06%), M3v (5.06%), M4 (8.86%), M5a (7.59%), M5b (1.27%), M6 (2.53%) and M7 (1.27%). ALL ; LB precursor (26.58%), LB common (6.33%), LT type (3.8%) and L3 in (2.53%). SBB is still an important and cheap method in diagnosis and classification of AML, while PAS can diagnose 60% of ALL. FCA not only helps in confirming morphologic diagnosis in AL but also helps in assigning specific lineage.

Keywords: Acute myeloid leukemia (AML), Acute lymphoid leukemia (ALL) Sudan Black B (SBB), Periodic Acid Schiff (PAS) , flowcytometric analysis (FCA)

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INTRODUCTION

DIAGNOSIS OF ACUTE LEUKEMIA:

The diagnosis of AL undergoes a stepwise approach. First is the distinction of an AL from other hematological neoplastic diseases and reactive disorders. Second is differentiating acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). The third step is the classification of AML and ALL into categories that define treatment and prognostic groups [1].

MORPHOLOGY AND CYTOCHEMISTRY IN THE DIAGNOSIS OF ACUTE LEUKEMIA:

In most cases, the first two facets of the diagnosis of AL can be achieved by careful morphological assessment of blood and bone marrow smears [2].

In AL, the morphologic identification of cells is sometimes difficult, due to a marked similarity between earlier precursors of different cells series. In cases of poorly differentiated AL, the morphologic features may be equivocal, requiring additional studies. In these cases the cytochemical stains are of great help in recognizing the type of precursor cells, especially when there is asynchronism between nuclear and cytoplasmic maturation. The presence of such cytochemically stainable components, in early precursors, indicates specific cellular differentiation, which the precursors are undergoing, thus making the identification easier. Sudan Black B, Periodic acid Schiff (PAS) are the most commonly used and is valuable in distinguishing AML from ALL. Distinct cytochemical patterns were observed in different types of leukemias [2]. SBB positive blasts are quite specific for AML.

PAS block positivity is seen in ALL, which has significance in absence of SBB positivity. Diffuse or granular PAS positivity has no significance. With the addition of cytochemistry to the morphologic assessment, most cases of acute leukemia can be appropriately designated as AML or ALL according to FAB classification. However, there remains a minority of cases that cannot be definitively diagnosed by these methods. In these cases the blastic cells are completely undifferentiated and cytochemistry cannot aid in the diagnosis since they have not yet developed their normal complement of enzymes and metabolic products [3].

IMMUNOPHENOTYPING IN THE DIAGNOSIS OF ACUTE LEUKEMIA:

The lineage of most cases of morphologically and cytochemically poorly differentiated AL can be accurately characterized by immuno phenotyping. Additionally, immunophenotypic subsets of AML and ALL can be determined [4,5]. Multiparametric flow cytometry is the preferred method for immunophenotyping AL.

The lineage of hematopoietic cells is defined both by antigens expressed and the absence of expression of antigens associated with a different lineage. Leukemia cells, however, may aberrantly express some antigens of another lineage or lack expression of an expected antigen [6]. It is important, therefore, to use panels that include sufficient numbers of antibodies to assess a spectrum of both myeloid and lymphoid antigens. The imunophenotying analyses must undergo some steps: lineage assignment, maturational analysis, complete characterization of blast cells and normal cells.

For ALLs, the immunophenotypic categories are particularly important because they identify distinctive treatment and prognostic groups .In AMLs, immunophenotyping is most important in distinguishing poorly differentiated cases from ALL [6].

FAB CLASSIFICATION OF ACUTE LEUKEMIA:

The FAB classification of AML is a lineage-based morphologic classification that categorizes cases according to the degree of maturation of the leukemic cells and their lineage differentiation and the bone marrow blast cell percentage must be at least 30% [1].

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WHO CLASSIFICATION OF ACUTE LEUKEMIA:

In the mid 1990s, the Society for Hematopathology in the United States and the European Association for Hematopathology were enlisted to update the WHO classification of hematopoietic neoplasms. This classification eliminates the problems of an exclusively lineage-based or an exclusively cytogenetic/molecular classification by combining the best features of both. The WHO classification of AMLs includes traditional FAB-type categories of disease, as well as additional disease types that correlate with specific cytogenetic findings and AML associated with myelodysplasia. The major categories of ALL in the revised WHO classification, defined by immunophenotype are three: B-cell precursor, T-cell precursor and mature B-cell ALL (Burkitt lymphoma leukemia). Within the B-cell precursor category there are several subtypes identified by cytogenetic/molecular abnormalities. The WHO classification has changed the grouping of ALL to reflect increased understanding of the biology and molecular pathogenesis of the diseases [7].

AIM OF THE STUDY:

The main aim of this study was to compare between morphologic and cytochemical diagnoses with flowcytometric diagnoses in acute leukemia.

MATERIALS AND METHODS

A total of 79 bone marrow specimens were diagnosed as acute leukemia based on morphology and cytochemistry over a period of 2 years.

The laboratory investigations analyzed included.

1. Examination of peripheral blood smears stained with Leishman's stain.
2. Examination of bone marrow aspirate smears (Leishman's).
3. Cytochemical stains, Sudan Black and Periodic acid Schiff (PAS).
4. FCA using primary and a secondary panel of monoclonal antibodies.

SUDAN BLACK B (SBB):

Is the most commonly used and the most valuable in distinguishing AML from ALL [8,9] This stain is specific for lipids, including neutral fat, phospholipids and sterols. Normal granulocyte precursors show increased sudanophilia that corresponds roughly to the number of granules. Promyelocytes contain a few sudanophilic granules, and mature polymorphonuclear neutrophils contain large numbers of sudanophilic granules [10].

PERIODIC ACID–SCHIFF (PAS):

Is a staining method used to detect polysaccharides such as glycogen, and mucosubstances such as glycoproteins, glycolipids and mucins in tissues. PAS staining can be used to assist in the diagnosis of several medical conditions [11].

FCA:

The primary panel of antibodies used were CD45 (for identifying blasts was added in all tubes), CD19, CD10, CD3, CD33, CD7, CD34, CD13. For some cases of AML (Acute myeloid leukemia) like AML M4/M5, CD14 and for AML M7, CD41 were also included in the primary panel. Secondary panel (CD117 and CD14) was used in few cases.

Data interpretation and reporting was done by reporting hematopathologists. Blasts were identified based on forward scatter and their dim expression of dim CD45. They also showed a strong positivity for CD34. More than 20% expression of any antigen was considered as positive. Acute lymphoblastic leukemias (ALL) were sub classified based on their expression of CD10, CD19, CD3 and CD7 where CD19 is a B-cell marker and, CD3 and CD7 are T-cell markers. AMLs were identified by their expression of CD13 and CD33. In suspected

monoblastic lineage leukemias, CD14 was used as an additional marker, AML M7 was included for which CD41 was also used in the primary panel.

RESULTS

Based on the morphology; 79 AL cases, 30 of them were classified as ALL and 46 were classified as AML, 3 cases remained unclassified [Table 1].

Table 1: Results of the cytochemical staining in these 79 AL cases.

Items	Sudan Black B		PAS		Total
	Positive	Negative	Positive	Negative	
ALL	0	30 (100%)	18 (60%)	12 (40%)	30 (37.9)%
AML	36 (78.2%)	10 (21.7%)	0	46	46 (58.2)%
AUL	0	3	0	3	3 (3.79)%
Total	36 (45.5%)	43 (54.4%)	18 (22.7%)	61 (77.2%)	79

Based on FAB classification of 79 acute leukemia cases; 58.21% were diagnosed as AML, 37.9 % were diagnosed as ALL, and 3.79% as AUL.

In AML, SBB was positive in 78.2 % of cases, these cases were sub classified as M1, M2, M3, M3v, M4 while 21.7% of cases had negative results and sub classified as M5a, M5b and M6. PAS were negative in all cases.

In ALL, PAS was positive in 60% of cases, and negative in 40% of cases. SBB was negative in all cases. AUL cases had negative results in both stains.
As shown in table (1).

AML classification by morphology and cytochemical stains were as follow: M1 (3.8%), M2 (25.32%), M3 (5.06%), M3v (5.06%), M4 (6.33%), M5a (8.86%), M5b (1.27 %) and M6 (2.53%). ALL classification, both L1 and L2 (35.44%), L3 (2.53%). As it shown in figure (1).

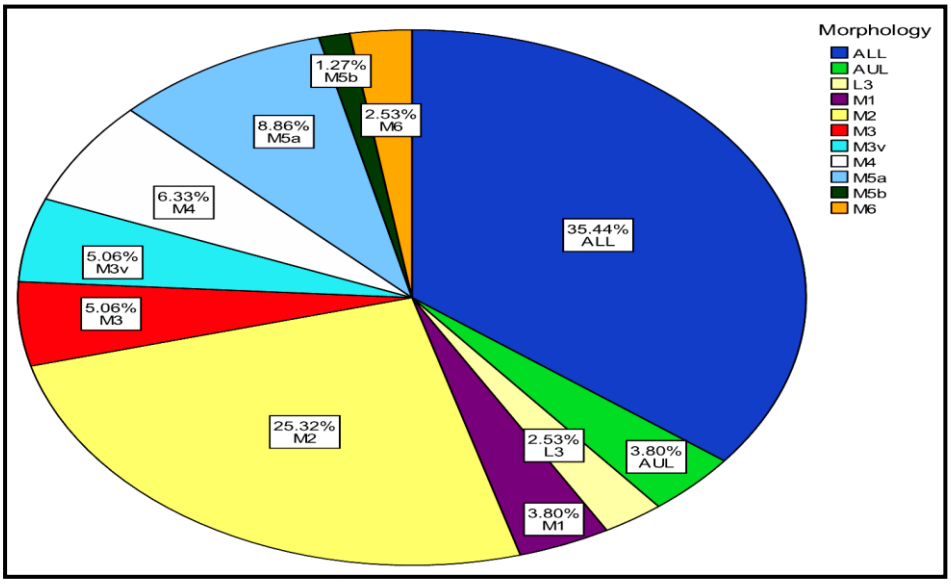

Figure (1): Percentage of morphological classification of 79 acute leukemia cases

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Flowcytometric diagnosis and classification of 79 leukemic cases were as follow:

AML; M0 (2.53%), M1(2.53%), M2 (24.05%), M3 (5.06%), M3v (5.06%), M4 (8.86%), M5a (7.59%), M5b (1.27%), M6 (2.53%) and M7 (1.27%).

ALL ; LB precursor (26.58%), LB common (6.33%), LT type (3.8%) and L3 in (2.53%). As it shown in figure (2).

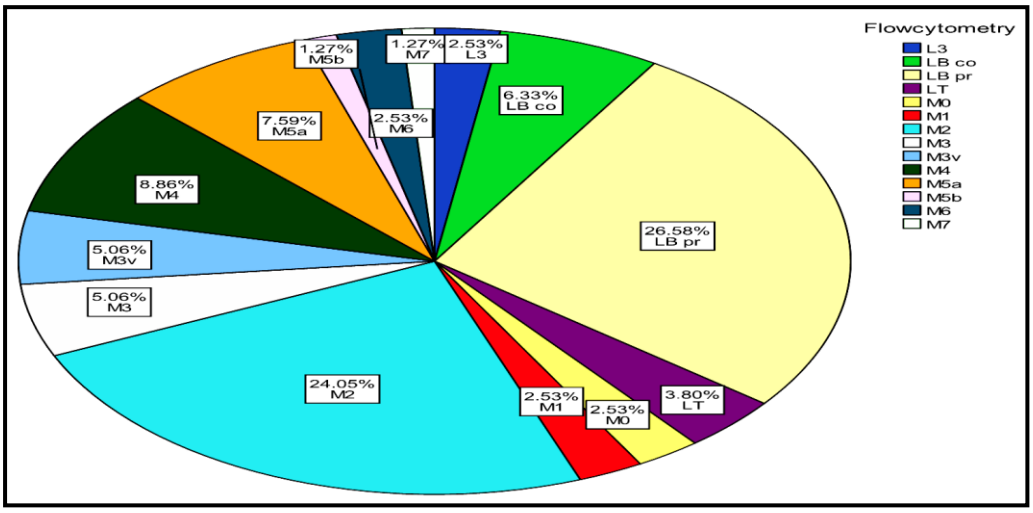

Figure (2): Percentage of Acute Leukemia Diagnosis by Flowcytometry

Table 2 represents the correlation of morphologic diagnosis versus flowcytometry diagnosis in the 79 cases; as this table shows that 30 cases diagnosed as ALL by morphology, proved by flowcytometry with provision of subdivision of leukemia which was unable by morphology and special stain alone, 2 of these ALL cases diagnosed as burkitt lymphoma ALL L3.

Table 2: Correlation of morphologic diagnosis versus flowcytometry diagnosis in the 79 cases

Morphology	No. of cases	Flowcytometry	No. of cases
ALL	28	LB common LB precursor L T	5 20 3
ALL L3	2	ALL L3	2
AUL	3	M0 M7	2 1
M1	2	M1 ALL	1 1
M2	21	M2 M4	19 2
M3	4	M3	4
M3v	4	M3v	4
M4	5	M4	5
M5a	7	M5a M4	6 1
M5b	1	M5b	1
M6	2	M6	2

Three cases out of 79 (3.7%)was undifferentiated and had negative both SBB and PAS cytochemical stains, flowcytometry solved the cases two as AML M0 and one as M7 . One M1 case, diagnosed by morphology out of 79 (1.2%) was diagnosed as ALL by flowcytometry. M3, M3v and M4 was fitted morphologically and by flowcytometry, cells had strong positivity of SBB with negative PAS, M5a , M5b and M6 diagnosed morphologically even with negative SBB.

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DISCUSSION

Amajor difficulty of the FAB classification is encountered in cases with negative cytochemical staining and in distinguishing M1 from L2 or M1 from M2 or M2 from M4.The WHO system has incorporated cytomorphology, immunophenotyping, cytogenetic and molecular changes. The result is a classification that enhances the clinical and prognostic relevance (Sushma Belurkar, et al 2013)[12]found that In analysis of 50 cases of acute leukemia Cytochemical analysis (PAS, SBB), when coupled with morphology rendered the diagnosis in > 80% of acute leukemia cases in a setting of lack of facilities for immunophenotyping, morphology and cytochemical analysis best serve the purpose in diagnosis of acute leukemia. While in the current study about 94.9% diagnosed as acute myeloid and lymphoid leukemia.

Also, Sushma Belurkar, et al stated that A total of 22/33 (66.7%) ALL cases and 11/12 (91.6%) AML cases could be assigned correct lineage based on morphology and cytochemical staining, and this results were agreed with current study results in which a total 20/30 ALL cases, 60% diagnosed with block positivity by PAS, and, 46/49 (93.9%) AML cases could be assigned correct lineage based on morphology and cytochemical staining.

Similar to the above results of the current study ; cytochemical stains used by Mhaweek et al.[13] included Sudan-black, specific esterase (alfa-naphthyl ASD chloroacetate esterase), non-specific esterase (alfa-naphthyl butyrate esterase), Periodic acid-Schiff, and acid phosphatase, definite diagnoses were made for all 10 of their AML cases, whereas diagnoses were possible in only 79.4% patients with ALL when only morphology and cytochemical staining was used.

However, in current study cytochemical staining is confidently useful in diagnosis and classification of AML cases, while in ALL cases was less valuable, this was agreed by Mhaweek et al. who stated that "Although cytochemical stains are essential to recognize the subtypes of AML, they are of limited use in differentiating the subtypes of ALL".

A similar study was done by Kheiri et al., [14] where they have compared cytochemical and flow cytometric diagnosis in 93 cases of acute leukemia. Their study has shown a lineage agreement of 95.8% of the cases. In their study, 89.2% of the myeloid leukemias showed agreement between cytochemical staining and FCA, whereas 80% of the ALL showed agreement between the two modalities, and these results were nearly in accordance in current study.

The observations in current study found that cytochemistry was of benefit in diagnosing AMLs where as in ALL, FCA was needed in about 40% of ALL cases could not be diagnosed based on cytochemical stains (PAS negative), thus these observations agreed with Sushma Belurkar, et al who stated that " cytochemistry was of good help in diagnosing AMLs where as in ALL, FCA was required in addition to morphology and cytochemistry as 1/3 rd of ALL cases could not be diagnosed based on cytochemical stains" [12].

A study was done at the TATA Memorial Hospital India, by Gujral et al., [15] and they concluded that FCA studies are important in those cases of acute myeloid leukemia where blasts do not show Auer rods and are negative for MPO and NSE stains. Thus the subtypes like AML-MO and, AML-M7, need FCA for a definitive diagnosis. Classical cases of AML do not require expensive FCA studies. These conclusions were agreed with current study in which 21.7% of AML cases showed negative SBB, and 3.09 % were diagnosed as AUL, in these cases FCA was helpful while 78.2 % were SBB positive and confidently diagnosed by morphology and cytochemical stains. these cases showed positive SBB with morphology of the blasts diagnosed as M1, M2, M3, M3v and M4 .

Only one case diagnosed as AML M1 by morphology with weak positive SBB, revealed to be ALL with apparent CD13, one possible explanation is that SBB stains a variety of lipids (neutral fat, phospholipids, sterol) in addition to primary granules and the positivity may be the result of altered lipid metabolism in leukaemic cells [16].

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CONCLUSIONS

SBB is still an important and cheap method in diagnosis and classification of AML, positive SBB with aid of morphology is confidently diagnostic for M1, M2, M3, M3v and M4 .
Positive PAS can diagnose 60% of ALL cases.

FCA not only helps in confirming morphologic diagnosis in acute leukemia but also helps in assigning specific lineage to the blasts, particularly in ALL.

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