CYTOCHEMICAL ANALYSIS IN LEUKEMIARajnikant Ahirwar¹, Rajendra Kumar Nigam², Reeni Malik³, Suhas Kothari⁴, Rubal Jain⁵**HOW TO CITE THIS ARTICLE:**

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ABSTRACT: Acute leukemias being a heterogeneous group of malignancies not only varying in clinical, morphologic, immunologic and molecular characteristics but also in prognosis and specific therapy. Currently flow-cytometry is considered the gold standard for diagnosis but it is costly and not available in majority of the centers in developing countries (Including our country), till then cheaper & easily performable alternative methods can be adapted for diagnosis. **AIM:** To correlate morphology and cytochemical findings to confirm the diagnosis of leukemia and to differentiate from leukemoid reaction. **MATERIAL AND METHODS:** After approval from institutional ethical committee 73 cases having provisional diagnosis of acute leukemia and leucoproliferative disorders (On the basis of leishman stained smears-Peripheral/bone marrow) were studied in the Department of Pathology, Gandhi Medical College, Bhopal from Oct 2013 to Nov 2014. Bone marrow smears stained for various cytochemical stains (MPO, PAS, NES, NAP). For all the special stains, commercially available kits (Leucognost) were used. **RESULT:** In our study only 71% cases were diagnosed on the basis of morphology only, but with addition of cytochemistry along with morphology diagnosis were made in 96% cases. 4% were undiagnosed even after cytochemistry requiring immunophenotyping, for which they were referred to the higher centers. **CONCLUSION:** In a setting of lack of facilities for immunophenotyping as in majority of centers in the underdeveloped and developing countries, morphology combined with cytochemical staining still serves the best purpose in diagnosis of acute leukemias. (More than 96% cases were diagnosed confirmatively in our series)

KEYWORDS: Cytochemistry, Leukemia.

INTRODUCTION: Acute leukemias are a heterogeneous group of malignancies with varying clinical, morphological, immunological and molecular characteristics. Many distinct types are known to carry predictable prognosis and warrant specific therapies. Distinction between lymphoid and myeloid leukemias is crucially important as treatment/prognosis differs a lot.

Examinations of peripheral smear/bone marrow aspirates using Romanowsky stained film at many times are not conclusive to identify the malignant cell line in leukemia.

Leukocyte cytochemistry encompasses the techniques which are used to identify diagnostically useful enzymes or other substances in the cytoplasm of haemopoietic cells, specially useful for the study of immature cells because conventional morphology as seen in Romanowsky stained films at times is very difficult to ascertain its differentiation features, thus they are particularly useful in identifying the lineage and stage of maturation of both normal and neoplastic haemopoietic cells.

Currently, flow cytometry is considered the gold standard for ascertaining the lineage of leukemic cells. In India, Flow Cytometry has been used mostly in the premier ICMR/CSIR/DBT/ National Aids Control Organization (NACO) research lab, few tertiary centers and private/highly specialized commercial centers.

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The cost varies depending upon the type of test for lymphoma-leukemia; starting from Rs. 3,500 and will increase further depending upon the panel used. Considering the fact that at present flowcytometry is either not available or beyond the capacity of poor patients in India, this study was carried out with the aim that cytochemistry will be more cost effective for diagnosis of leukemia. Leishman's stained peripheral blood/Bone marrow smears of the leukemia and suspected leukemia were stained with special cytochemical stains viz; myeloperoxidase, periodic acid schiff, alkaline phosphates and non-specific esterase.

MATERIAL AND METHODS: The study was conducted after approval from institutional ethical committee in the Department of Pathology, Gandhi Medical College, Bhopal from Oct 2013 to Nov 2014.

73 cases were selected from in and out patient departments of Gandhi Medical College and associated Hamidia Hospital Bhopal having provisional diagnosis of acute leukemia and leucoproliferative disorder so on basis of leishman stained smears-Peripheral/bone marrow. Thorough case history and clinical examination as per predefined Performa with Hemoglobin estimation, WBC count, Platelet count and various hematological indices were evaluated using automated analyzer.

Peripheral blood/bone marrow smear was made on clean glass slides with fresh blood samples; smears were fixed and stained by leishmans stain. Smears prepared on clean glass slides, fixed by methanol and stained for various cytochemical stains (MPO, PAS, NES, NAP). For all the special stains, commercially available kits (Leucognost) were used.

OBSERVATIONS: The present study comprises 73 cases of suspected Leukemia taken from Department of Pathology, Gandhi Medical College, and associated Hamidia Hospital Bhopal between Oct. 2013 to Nov. 2014. The observations made in this study are as follows.

Type of Leukemias	No. of Patients	Percentages
ALL	23	31.51%
AML	11	15.07%
CML	35	47.95%
CLL	1	1.37%
Undiagnosed	3	4.11%
Total	73	

Table 1: Type of Leukemias

Out of 73 cases on final diagnosis, 23 cases (31.51%) were of Acute Lymphoblastic Leukemias, 11 cases (15.07%) were of Acute Myeloblastic Leukemias, 35 cases (47.97%) were of Chronic Myeloid Leukemias, 1 case (1.37%) was of Chronic Lymphocytic Leukemia and 3 cases (4.11%) remained undiagnosed.

Sex	No. Of Patients	Percentages
Male	31	42.47%
Female	42	57.53%
Total	73	100%

Table 2: Sex Distribution

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Out of 73 cases - 31cases (42.47%) were Male and 42cases (57%) were Female.

Age in years	No. of patients	Percentage
0 - 15	18	24.66%
16 - 30	21	28.77%
31 - 45	19	26.03%
>45	15	20.55%
Total	73	

Table 3: Age Distribution

Out of 73 cases – 18 cases (24.66%) were of age group 0 to 15 years, 21 cases (28.77%) were of age group 16 to 30 years, 19 cases (26.03%) were of age group 31 to 45 years and 15 cases (20.55%) were of above 45 years.

Age in Years	No of patent							
	ALL		AML		CML		CLL	
0 -15	13	56.52%	2	18.18%	3	8.57%	0	0
16 - 30	5	21.74%	4	36.36%	9	25.71%	0	0
31 - 45	3	13.04%	4	36.36%	13	37.14%	0	0
> 45	2	8.70%	1	9.09%	10	28.57%	1	100%

Table 4: Age distribution of cases

Acute Lymphoblastic Leukemia is more common (56.52%) in age group of 0 to 15 years.

Acute Myeloblastic Leukemia, Chronic Myeloid Leukemia and Chronic Lymphocytic Leukemia are more common 9(81.81%), 32(91.42%) and 1(100%) in age group above 15 years.

Sex	ALL		AML		CML		CLL	
Male	15	65.22%	4	36.36%	10	28.57%	0	0
Female	8	34.78%	7	63.64%	25	71.43%	1	100.00%

Table 5: Sex distribution of cases

Acute Lymphoblastic Leukemia showed male (65.22%) preponderance whereas Acute Myeloblastic Leukemia, Chronic Myeloid Leukemia showed female (63.64%), (71.43%) preponderance. Only one female case of Chronic Lymphocytic Leukemia was seen.

Hb in gram%	ALL		AML		CML		CLL	
< 6	13	56.52%	8	72.73%	2	5.71%	0	0
6.1 - 9	9	39.13%	3	27.27%	12	34.29%	0	0
9.1 - 12	1	4.35%	0	0	18	51.43%	1	100.00%
Above 12	0	0	0	0	3	8.57%	0	0

Table 6: Distribution of Hemoglobin in cases

In Acute Lymphoblastic Leukemia and Acute Myeloblastic Leukemia most of the cases (56.52% and (72.73%) had less than 6 gm% Hb. In Chronic Myeloid Leukemia only 5.71% cases had

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severe anemia, while 34.29% and 51.43% had moderate and mild anemia respectively. In Chronic Lymphocytic Leukemia patient had mild anemia.

TLC	ALL		AML		CML		CLL	
Less than 4000	9	39.13%	7	63.64%	1	2.86%	0	0
4001-11000	6	26.09%	0	0	2	5.71%	0	0
11001-50000	6	26.09%	2	18.18%	9	25.71%	1	100.00%
50001-100000	0	0	0	0	5	14.29%	0	0
100001-200000	1	4.35%	2	18.18%	6	17.14%	0	0
Above 200000	1	4.35%	0	0	12	34.29%	0	0

Table 7: Total Leukocyte Count in cases

In Acute Lymphoblastic Leukemia and Acute Myeloblastic Leukemia 39.13% and 63.64% of cases showed count less than 4000 /cu mm respectively. While in Chronic Myeloid Leukemia most of cases (91.43%) showed leukocytosis.

Platelet Count/cu mm	ALL		AML		CML		CLL	
<50000	16	69.57%	11	100.00%	1	2.86%	0	0
50000-1 lac	3	13.04%	0	0	0	0	0	0
1lac- 1.5lacs	1	4.35%	0	0	1	2.86%	0	0
Above 1.5 lacs	3	13.04%	0	0	33	94.29%	1	100.00%

Table 8: Platelet Count in cases

Most of the cases (82.61% and 100%) of Acute Leukemia had moderate to severe thrombocytopenia. Whereas in Chronic Leukemia most of cases had platelet above 1lakh/cu mm.

Sub-classification	No. of patent	Percentage
AML(M1)	2	18.18%
AML(M2)	1	9.09%
AML(M3)	4	36.36%
AML(M5)	1	9.09%
AML(M6)	1	9.09%
AML(M7)	2	18.18%
Total	11	

Table 9: Sub-classification Acute Myeloid Leukemia

Most common subtype of Acute Myeloid Leukemia was M3 subtype which comprised 36.36% of cases.

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Final diagnosis	Diagnosis on leishman stain	No. of cases	Negative on MPO stain	Positive on MPO stain	Negative on PAS stain	Positive on PAS stain	Low (<10) score on NAP stain	Positive on NSE Stain
AML(11)	AML	7	0	7	7	0	0	0
	?AML	4	0	3	4	0	0	1
ALL(23)	ALL	12	12	0	0	12	0	0
	?ALL	11	11	0	0	11	0	0
CML(35)	CML	32	0	32	32	0	32	0
	?CML	3	0	3	3	0	3	0
CLL(1)	CLL	1	1	0	0	1	0	0
Undiagnosed(3)		3	3	0	3	0	0	0

Table 10: Number of cases with cytochemical positive staining

Myeloblast of AML and CML showed positive (coarse brown black granules) staining with MPO in more than 3% of blasts in all cases except one in which NSE stain was positive. Whereas ALL and CLL were negative for MPO stain. The lymphoblast of ALL and CLL showed positive (block positive) PAS staining. Whereas myeloblasts of AML and CML were negative. The NAP scoring was done in all (35) cases of CML and the range of NAP score was 0 to 10. Undiagnosed 3 cases were negative for all (MPO, PAS, NSE) stain.

Diagnosis	By morphology only	By morphology with cytochemistry
ALL	12	23
AML	7	11
CML	32	35
CLL	1	1
Undiagnosed	21	3

Table 11: Distribution of cases (morphology only v/s morphology with cytochemistry)

Table shows only 52 cases (71%) were diagnosed on the basis of morphology only. But with use of cytochemistry along with morphology 70 cases (96%) were diagnosed. 3 cases (4%) were undiagnosed even after cytochemistry and for which immunophenotyping is required.

DISCUSSION: In present study 73 cases of leukemias were studied 34 cases (46.58%) and 36 cases (49.32%) were of acute and chronic leukemias respectively and 3 cases (4.11%) were undiagnosed.

Out of which 23 cases (31.51%) were of Acute Lymphoblastic Leukemias, 11 cases (15.07%) were of Acute Myeloid Leukemias, 35 cases (47.97%) were of Chronic Myeloid Leukemias, 1 case (1.37%) was of Chronic Lymphocytic Leukemia.

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Reference	Region (PERIOD OF STUDY)	No. of cases	ALL	AML	CML	CLL
Chatterjee et al ¹	Calcutta (1949–1961)	544	22.5	32.5	35.9	5.9
Advani et al ²	Mumbai (1960–1975)	1126	30	13	40	9
Prakash et al ³	Pondicherry (1970–1979)	278	35	29.5	30.8	3.2
Rani et al ⁴	Delhi (1970–1979)	490	15.5	30.8	45.3	5.7
Verghese et al ⁵	Kerala (1980–1983)	1016	39.2	19.6	16.4	1.9
Kushawaha et al ⁶	Lucknow (1971–1984)	970	9.3	38.7	48	2.6
Shome et al ⁷	Chandigarh (1975–1983)	820	24	29.3	36.7	8.8
Dicosta et al ⁸	Mumbai (1975–1984)	242	36	22	38	2
Rathee et al ⁹	Haryana (2008-2012)	650	17.2	33.8	39	10
Our study	Bhopal (2013-2014)	73	31.51	15.07	47.97	1.37

Frequency (in percentage) of various Leukemias in India

In our study predominant cases were of CML (47.97%) which correlates with Chatterjee et al, Advani et al, Rani et al, Kushawaha et al, Shome et al, Dicosta et al, Rathee et al study. However Prakash et al and Verghese et al show ALL predominance which could be due to population bias. The second predominant cases were of ALL (31.51%) in our study which correlates with study of Advani et al and Dicosta et al. In present study Acute Lymphoblastic Leukemia was more common (56.52%) in children than Acute Myeloid Leukemias (18.18%). Same was observed by Neglia JP et al (1988).¹⁰ and Ribera JM et al (2009).¹¹

Reference	Study
Neglia, jp, and Robison. ¹⁰ 1988	In children, acute lymphoblastic leukemia (80%) is more common than acute myeloid leukemia
Ribera JM, Oriol A. ¹¹ 2009	Acute lymphoblastic leukemia (ALL) is the most frequently diagnosed malignancy in children, representing nearly one third of all pediatric cancers
Our study	Acute Lymphoblastic Leukemia is more common (56.52%) in age group of 0 to 15 years

In Acute Myeloblastic Leukemias an adult predominance (81.81%) was observed in this study which correlates with study of Boros, L. and Bennett, J.M,¹² Khalid Hassan Nadeem Ikram, Sajid Hussain Shah.¹³ and Greer John P, Baer Maria.R, Kinney Marsha.C.¹⁴

Reference	Study
Boros, L. and Bennett, J. M (1984) ¹²	AML occurs twice as often as ALL, the vast majority of cases occurring in adults
Khalid Hassan Nadeem Ikram, Sajid Hussain Shah ¹³ (1994)	AML was observed more commonly in adults (79%) as compared to children (21%), whereas ALL was commoner in children (72%) as Compared to in 28% amongst adults.
Greer John P, Baer Maria. R, Kinney Marsha. C (2009) ¹⁴	AML accounts for less than 15% of cases of leukemia in children below 10years, 25-30% between 10-15 years, and in adults, it accounts for 80-90% of cases of acute leukemias.
Our study	Acute Myeloblastic Leukemias an adult predominance (81.81%)

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In Acute Lymphoblastic Leukemia and Acute Myeloblastic Leukemia most of the cases (56.52%) and (72.73%) had severe anemia. In Chronic Myeloid Leukemia only 5.71% cases had severe anemia, while 34.29% and 51.43% had moderate and mild anemia respectively. In Chronic Lymphocytic Leukemia patients had mild anemia.

FAB type	Sultan et al (250) 1981¹⁵	Miguel et al (120) 1986¹⁶	Chessells* et al (112) 1986¹⁷	Alvi et al (26) 1990¹⁸	Chaudhry et al (54) 1993¹⁹	Khalid Hassan (81) 1994¹³	Present Study⁽¹¹⁾
M1	21	13	10	15.3	13	18.5	18.18
M2	32	14	26	35	44.4	23.9	9.09
M3	16	14	6	15.3	11.1	11.1	36.36
M4	16	22	22	19.2	24	29.6	0
M5	12	21	24	11.5	3.7	11.1	9.09
M6	3	7	9	3.7	3.7	1.2	9.09
M7	0	0	0	0	0	1.2	18.18

Table compares the distribution of FAB types of AML in the present study with some previous studies (in percentage)

*study conducted on childhood AML.

Sub classification of AML was done which showed 36.36% cases of M3, 18.18% cases of M1 and M7, 9.09% cases of M2, M5, and M6. In present study there were no cases belonging to M0 and M4 subtype which not correlates with any of above mention studies it may be due to number of cases. In present study four special staining procedures were done after making a provisional diagnosis (on morphology) of leukemias by leishman stain. Stain used Myeloperoxidase, Periodic acid Schiff, Nonspecific Esterase and alkaline phosphates. On special staining we found Myeloblasts of AML and CML showed positive (coarse brown black granules) staining with MPO in more than 3% of blasts in all cases except one in which NSE stain was positive. Whereas ALL and CLL were negative for MPO stain.

The lymphoblasts of ALL and CLL showed positive (block positive) PAS staining. Whereas myeloblasts of AML and CML were negative. The NAP scoring was done in all (35) cases of CML and the range of NAP score was 0 to 10. Undiagnosed 3 cases were negative for all (MPO, PAS, NSE) stain. They were advised for flow-cytometry, immunophenotyping for which they were referred to higher center.

Diagnosis	By morphology only	By morphology with cytochemistry
ALL	12	23
AML	7	11
CML	32	35
CLL	1	1
Undiagnosed	21	3
Morphology only v/s Morphology with cytochemistry		

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In our study out of 73 cases 52 cases were diagnosed on the basis of morphology only, but with combined use of cytochemistry along with morphology 70 cases (96%) were diagnosed. 3 cases (4%) were undiagnosed even after cytochemistry, for which immunophenotyping is required and they were referred to the higher centers. This is similar to Belurkar S et al.²⁰ [Cytochemical analysis of leukemia by (PAS, MPO, SBB), when coupled with morphology rendered the diagnosis in >80% of our acute leukemia cases.], Mhaweek et al.²¹ [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 were used.]

Thus in a setting of lack of facilities for immunophenotyping as in majority of centers in the underdeveloped and developing countries, morphology combined with cytochemical staining still serves the best purpose in diagnosis of acute leukemias. (More than 96% cases were diagnosed confirmatively in our series.

CONCLUSION: Acute leukemias being a heterogeneous group of malignancies varying in clinical, morphologic, immunologic and molecular characteristics and also in prognosis & specific therapy. Flow-cytometry is considered the gold standard for diagnosis but being costly and not available in majority of the centers in developing countries including India, so alternative methods which are cheaper and easily performable can be adapted for diagnosis. In the present study only 71% cases (52 out of 73) were diagnosed on the basis of only morphology in which 12 cases (16%) of ALL, 07 cases (10%) of AML, 32cases (44%) of CML, 01case (1%) of CLL while combined with cytochemistry 96% cases (70 out of 73) were diagnosed, thus 18 more cases were diagnosed with addition of cytochemistry out of which 11, 04 & 03 cases were of ALL, AML & CML respectively.

Thus cytochemical analysis coupled with morphology can serve the purpose in the diagnosis of leukemias till immune-phenotyping and cytogenetics becomes available for everyone.

Figure 1: Bone marrow smear of AML (M2) showing MPO positive myeloblast (Oil immersion -100x)

Figure 1

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Figure 2: Bone marrow smear of ALL (L2) showing PAS positive Lymphoblast of variable size (oil immersion -100x)

Figure 2

Figure 3: Peripheral smear showing alkaline phosphatase positive neutrophils (Oil immersion-100x).

Figure 3

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