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Original Article

Pathology Section

Evaluation of Morphocytochemistry and Flow Cytometry in Acute Leukaemia: A Cross-sectional Study

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ABSTRACT

Introduction: Acute leukaemias are rapidly progressive haematological malignancies that require timely and accurate lineage classification to guide appropriate treatment. Morphology and cytochemical staining have traditionally played a central role in diagnosis, particularly in resource limited settings, while flow cytometry based immunophenotyping is currently regarded as the gold standard for definitive lineage assignment because of its superior diagnostic precision. Evaluating the agreement between these diagnostic modalities remains important for optimising diagnostic workflows.

Aim: To compare the diagnostic performance and concordance of Morphocytochemistry (MCC) with Flow Cytometry Immunophenotyping (FCM) in the classification of acute leukaemias.

Materials and Methods: The present prospective observational study included 169 newly diagnosed cases of acute leukaemia evaluated over a two-year period from November 2015 to October 2017 at a Tertiary Health Care Centre in Central India. Peripheral blood and/or bone marrow samples were examined using morphology and cytochemical stains, including Myeloperoxidase (MPO) and Periodic Acid-Schiff (PAS). FCM using a multiparametric antibody panel was regarded as the gold standard for definitive diagnosis. Diagnostic accuracy parameters were calculated, and agreement between MCC and flow cytometry was assessed using Cohen's kappa statistic.

Results: Flow cytometry classified 77 (45.29%) cases as Acute Myeloid Leukaemia (AML), 52 (30.58%) cases as B-lineage Acute Lymphoblastic Leukaemia (ALL), and 40 (23.52%) cases as T-lineage ALL. PAS staining was positive in 55 of 92 ALL cases, demonstrating high specificity (97.4%) for lymphoid lineage, while MPO was positive in 60 of 77 AML cases, showing 100% specificity for myeloid lineage. MCC misclassified 12 AML cases and 15 ALL cases that were correctly categorised by flow cytometry. Overall agreement between the two diagnostic modalities was substantial (κ=0.685, $p<0.001$).

Conclusion: MCC remains a valuable preliminary diagnostic tool in acute leukaemia, particularly in resource-limited settings. However, FCM provides superior diagnostic accuracy and serves as the definitive method for lineage determination. An integrated diagnostic approach combining morphology, cytochemistry, and immunophenotyping ensures optimal classification of acute leukaemias.

Keywords: Definitive lineage assessment, Diagnostic performance, Myeloperoxidase, Periodic acid-schiff

INTRODUCTION

Acute leukaemias represent a heterogeneous group of haematologic malignancies characterised by uncontrolled proliferation of immature haematopoietic precursors, resulting in bone marrow failure and rapid clinical deterioration [1]. Early and accurate diagnostic subclassification into AML, ALL, or Mixed-Phenotype Acute Leukaemia (MPAL) is essential, as treatment protocols and prognostic determinants differ significantly between these entities. Contemporary diagnostic frameworks, including the 2022 World Health Organisation (WHO) classification and the 2022 International Consensus Classification, emphasise a multiparametric approach integrating morphology, cytochemistry, immunophenotyping, cytogenetics and molecular abnormalities [2,3].

The MCC, historically a cornerstone of leukaemia diagnosis, utilises cytochemical stains-such as MPO, Sudan Black B, and non specific esterases- to support lineage assignment, particularly in distinguishing myeloid from lymphoid and monocytic differentiation [4]. Although MCC remains inexpensive and accessible, its diagnostic accuracy is limited by subjective interpretation, inability to detect aberrant antigen expression, and reduced utility in genetically defined subtypes increasingly recognised in modern classification systems [5].

The FCM has emerged as a highly sensitive modality capable of simultaneous multiparametric evaluation of lineage-associated antigens, enabling refined classification, identification of MPAL, and detection of Minimal Residual Disease (MRD) [6-8]. Despite its advantages, access to FCM may be restricted in resource-constrained settings, and real-world comparative data examining the concordance between MCC and FCM- especially in newly diagnosed acute leukaemias- remain limited [9,10]. Understanding the degree of agreement between these methods is crucial for centers where MCC continues to guide initial diagnosis.

The present study is justified by the widespread use of MCC alongside the rising global reliance on FCM for acute leukaemia diagnosis. While FCM offers detailed immunophenotyping, MCC remains valuable in resource-limited settings. Comparing these methods is essential to assess diagnostic concordance, misclassification, and the impact of discordant results. Hence the present study aimed to compare the diagnostic performance of MCC and flow cytometry and to determine the concordance between the two methods for lineage and subtype classification in acute leukaemia.

MATERIALS AND METHODS

The present cross-sectional study was conducted in the Department of Pathology, Government Medical College, Nagpur, Maharashtra, India over a two-year period from November 2015 to October 2017. Ethical approval was obtained from the Institutional Ethics Committee (IEC No.: 824 EC/Pharmac/GMC/NGP).

Sample size calculation: Sample size was calculated assuming an expected concordance of 68.5% between MCC and flow cytometry, as reported by Mahto SK and Prasad N with a 95% confidence level

and 8% absolute precision [9]. The minimum required sample size was estimated to be 130 cases. After adjusting for an anticipated attrition rate of approximately 30%, the final target sample size was set at 170 cases.

Inclusion and Exclusion criteria: Inclusion criteria consisted of all newly diagnosed or first-presentation patients presenting with clinical features suggestive of acute leukaemia, supported by peripheral smear or bone marrow findings demonstrating ≥20% blasts. Exclusion criteria included biphenotypic leukaemia, relapsed or previously treated leukaemia cases, chronic myeloid leukaemia in blast crisis, cases with incomplete clinical or laboratory data, and samples in which flow cytometry could not be performed.

Study Procedure

Sample collection and preparation: Approximately, 2-3 mL of peripheral blood was collected in Ethylenediamine Tetraacetic Acid (EDTA) vials for complete blood count analysis and processed using an automated haematology analyser (ERMA). Peripheral smears were prepared from fresh capillary blood or anticoagulated venous blood [11]. Bone marrow aspiration was undertaken in patients presenting with pancytopaenia, unexplained cytopaenias, or leukocyte counts <4,000/µL [12]. Bone marrow aspirates or peripheral blood samples were processed immediately for flow cytometry using the lyse-stain-wash protocol, with cell concentrations adjusted to approximately 10,000 cells/µL, following standard haematopathology guidelines [13].

Cytochemistry and morphological evaluation: Air-dried smears were stained using Leishman, MPO, and PAS stains for morphological and cytochemical evaluation. Findings were interpreted according to established morphological criteria for acute leukaemia classification [13,14].

Immunophenotyping and flow cytometry (gold standard): Immunophenotyping was regarded as the gold standard for definitive lineage assignment and subtyping. A panel of 20 monoclonal antibodies conjugated to fluorochromes such as Fluorescein Isothiocyanate (FITC), Phycoerythrin (PE), and Allophycocyanin (APC) was used for surface and cytoplasmic antigen staining. Antibody cocktails were titrated for optimal resolution of haematopoietic lineages. Analyses were performed on a Beckman Coulter Navios flow cytometer equipped with three lasers and 10-colour capability. A minimum of 50,000 ungated list-mode events were acquired. Data were analysed using Kaluza software, following standardised gating strategies and predetermined dot-plot sequences.

Quality assurance: Daily quality assurance of the flow cytometer was performed using commercial calibration beads. Photomultiplier tube settings, instrument stability, and fluorescence sensitivity were monitored, with results verified against target ranges using Levy-Jennings plots to ensure reproducibility and accuracy of data.

STATISTICAL ANALYSIS

Data analysis was conducted from November to December 2017 using Epi Info version 7.2. Quantitative variables were reported as means with standard deviations, while categorical variables were expressed as frequencies and percentages. Diagnostic accuracy parameters-including sensitivity, specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV), and overall diagnostic accuracy were calculated using immunophenotyping as the reference standard. Statistical comparisons employed the Chi-square test, Fisher's-exact test, and unpaired t-tests. Concordance between MCC and flow cytometry was evaluated using Cohen's kappa statistic, with $p<0.05$ considered statistically significant.

Quality assurance: Daily quality assurance of the flow cytometer was performed using commercial calibration beads. Photomultiplier tube settings, instrument stability, and fluorescence sensitivity were monitored, with results verified against target ranges using Levy-Jennings plots to ensure reproducibility and accuracy of data.

RESULTS

The study included immunophenotypic distribution of 169 study subjects with acute leukaemia, comprising 92 (54.4%) ALL and 77 (45.6%) AML cases. A significant difference in age distribution was observed between ALL and AML ($p <0.001$). ALL predominantly affected younger patients, with 47.83% (44/92) in the 0-10-year age group and 23.91% (22/92) aged 11-20 years. In contrast, AML was more common in older age groups, particularly >60 years (20.78%, 16/77). Males constituted 103/169 (60.96%) and females 66/169 (39.05%). Although males predominated in both ALL (66.30%) and AML (54.54%), the gender difference was not statistically significant ($p=0.12$).

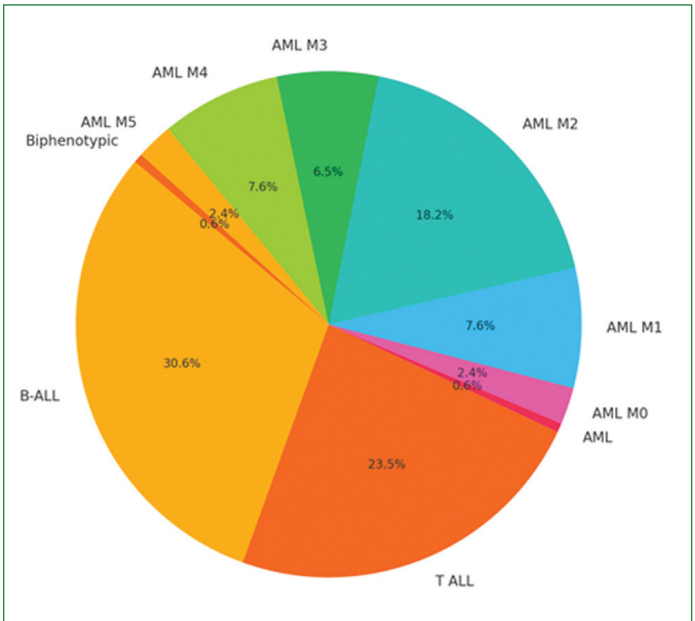
With respect to clinical presentation, fever was the most common symptom, reported in 64 patients (69.56%) with ALL and 41 patients (53.24%) with AML, accounting for 105 cases (62.13%) overall. Bleeding tendency was more frequently observed in AML (29 patients, 37.66%) compared to ALL (20 patients, 21.73%). Hepatomegaly and splenomegaly were common findings, with liver enlargement seen in 76 patients (44.97%) and splenomegaly in 67 patients (39.64%). Lymphadenopathy was notably more prevalent in ALL, affecting 54 patients (58.69%), compared to 15 patients (19.48%) with AML. Mediastinal mass was predominantly associated with ALL, observed in 12 patients (13.04%), and was rare in AML (2 patients, 2.59%). Pain, including abdominal or joint pain, was reported more commonly in ALL (60 patients, 65.21%) than in AML (16 patients, 20.77%) [Table/Fig-1].

Variables	ALL (n=92) n (%)	AML (n=77) n (%)	Total n (%)	Statistical Test	p-value	Significance
Age group (years)						
0-10	44 (47.83)	10 (12.99)	54 (31.95)	Chi-square test	<0.001	*
11-20	22 (23.91)	7 (9.09)	29 (17.16)			
21-30	8 (8.70)	16 (20.78)	24 (14.20)			
31-40	13 (14.13)	11 (14.29)	24 (14.20)			
41-50	2 (2.17)	7 (9.09)	9 (5.33)			
51-60	2 (2.17)	10 (12.99)	12 (7.10)			
>60	1 (1.09)	16 (20.78)	17 (10.06)			
Gender						
Male	61 (66.30)	42 (54.54)	103 (60.96)	Chi-square test	0.12	
Female	31 (33.69)	35 (45.45)	66 (39.05)			
Bleeding tendency	20 (21.73)	29 (37.66)	49 (28.99)	Chi-square test	0.03	*
Hepatomegaly	47 (51.08)	29 (37.66)	76 (44.97)	Chi-square test	0.08	
Splenomegaly	38 (41.30)	29 (37.66)	67 (39.64)	Chi-square test	0.63	
Lymphadenopathy	54 (58.69)	15 (19.48)	69 (40.82)	Chi-square test	<0.001	*

Mediastinal mass	12 (13.04)	2 (2.59)	14 (8.28)	Fisher's exact test	0.01	*
Fever	64 (69.56)	41 (53.24)	105 (62.13)	Chi-square test	0.03	*
Weakness	34 (36.95)	32 (41.55)	66 (39.05)	Chi-square test	0.54	
Pain (abdominal/joint)	60 (65.21)	16 (20.77)	76 (44.97)	Chi-square test	<0.001	*

[Table/Fig-1]: Demographic and clinical parameters of study subjects (N=169)$.
Values are expressed as number and percentage. Chi-square test was used for categorical variables; Fisher's exact test was applied where expected cell counts were <5. p<0.05 was considered statistically significant. $Total cases were 169, since one case was biphenotypic.

The immunophenotypic distribution of the 170 study subjects showed that B-cell ALL (B-ALL) was the most common subtype, observed in 52 patients (30.6%), followed by T-cell ALL (T-ALL) in 40 patients (23.5%). Among AML subtypes, AML M2 was seen in 31 cases (18.2%), AML M1 in 13 cases (7.6%), AML M4 in 13 cases (7.6%), and AML M3 in 11 cases (6.5%). Less frequent subtypes included AML M5 in 4 patients (2.4%), AML M0 in 1 patient (0.6%), and biphenotypic acute leukaemia in one patient (0.6%). Overall, lymphoid leukaemias accounted for a higher proportion of cases compared to myeloid leukaemias, with a heterogeneous distribution across AML subtypes [Table/Fig-2].



[Table/Fig-2]: Distribution of study subjects based on immunophenotyping (n=170).

The diagnostic performance of cytochemical stains in differentiating ALL and AML is summarised as follows. PAS staining was positive in 55 cases of ALL (59.78%) and in two cases of AML (2.60%), while PAS negativity was observed in 37 ALL cases (40.22%) and 75 AML cases (97.40%). PAS demonstrated a sensitivity of 59.78% and a specificity of 97.40% for the diagnosis of ALL, with a PPV of 96.49% and a NPV of 66.95%.

The MPO staining showed positivity exclusively in AML, with 60 AML cases (77.92%) being MPO positive and no positive cases among ALL (0%). MPO negativity was observed in 92 ALL cases (100%) and 17 AML cases (22.08%). MPO exhibited a sensitivity of 77.92% and a specificity of 100% for the diagnosis of AML, with both PPV and specificity being 100%, and an NPV of 84.40%. Overall, cytochemical staining- particularly MPO- showed high diagnostic accuracy in distinguishing myeloid from lymphoid leukaemias [Table/Fig-3].

The table compares morphological diagnosis with PAS and MPO cytochemical staining patterns in patients with acute leukaemia. Among cases initially classified as acute leukaemia (n=66), PAS positivity was observed in 25 cases (37.9%) and PAS negativity in 41 cases (62.1%), while MPO positivity was seen in 13 cases (19.7%) and MPO negativity in 53 cases (80.3%). Based on combined morphocytochemical evaluation, 28 cases remained undifferentiated, whereas 25 cases were reclassified as ALL and 13 cases as AML. In morphologically diagnosed ALL cases (n=53), PAS positivity was noted in 30 cases (56.6%) and PAS negativity in 23 cases (43.4%). None of the ALL cases showed MPO positivity, with all 53 cases (100%) being MPO negative. Morpho-cytochemical diagnosis confirmed all these cases as ALL. Among morphologically diagnosed AML cases (n=51), PAS positivity was rare, seen in only two cases (3.9%), while 49 cases (96.1%) were PAS negative. MPO positivity was observed in 47 cases (92.2%), with four cases (7.8%) being MPO negative. All cases in this group were confirmed as AML on morpho-cytochemical assessment. Overall, the table highlights the complementary role of PAS and MPO staining in refining the diagnosis of acute leukaemia and resolving initially undifferentiated cases [Table/Fig-4].

The table presents a comparison between flow cytometry and morphocytochemistry diagnoses in 169 patients with acute leukaemia. Among patients diagnosed as AML by flow cytometry (n=77), 63 cases (81.8%) showed concordant AML diagnosis on morphocytochemistry, while 12 cases (15.6%) were categorised as acute leukaemia and two cases (2.6%) as ALL. Among patients diagnosed as ALL by flow cytometry (n=92), concordant ALL diagnosis on morphocytochemistry was observed in 76 cases (82.6%). The remaining cases included 15 patients (16.3%) classified as acute leukaemia and one patient (1.1%) as AML on morpho-cytochemical evaluation. Overall, morphocytochemistry classified 27 cases (16.0%) as acute leukaemia, 64 cases (37.9%) as AML, and 78 cases (46.2%) as ALL. There was substantial agreement between flow cytometry and morphocytochemistry, as indicated by a Cohen's kappa coefficient of 0.685. This agreement was statistically significant, with a p-value of <0.001, demonstrating good concordance between the two diagnostic modalities in the classification of acute leukaemias [Table/Fig-5].

The table summarises the expression patterns of a comprehensive panel of immunophenotypic markers in patients with acute leukaemia, categorised into AML, B-ALL and T-ALL.

CD45 was expressed in all AML and T-ALL cases (100% each) and in the majority of B-ALL cases (96.15%). The stem cell marker CD34 showed variable expression, being most frequent in AML (64.93%), followed by B-ALL (57.69%) and T-ALL (45.0%). Myeloid lineage–associated markers such as CD13, CD33, CD117, and cytoplasmic MPO (cMPO) were predominantly expressed in AML, with CD13 and CD33 positivity exceeding 90% and cMPO expressed in 89.61% of cases, while these markers were largely absent or minimally expressed in lymphoid leukaemias.

B-ALL demonstrated a characteristic B-lineage immunophenotype, with uniform expression of CD19 and cytoplasmic CD79a (100% each) and high expression of CD10 (92.30%) and HLA-DR (94.23%). In contrast, T-ALL showed strong expression of T-cell-associated markers, including cytoplasmic CD3 (100%), CD7 (97.50%), CD1a (57.50%), CD4 (50.0%), and CD8 (57.50%), confirming T-lineage differentiation [Table/Fig-6].

Cytochemistry	ALL positive (n)	AML positive (n)	ALL negative (n)	AML negative (n)	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)
Periodic Acid Schiff (PAS)	55	2	37	75	59.78	97.4	66.95	96.49
Myeloperoxidase (MPO)	0	60	92	17	77.92	100.0	84.4	100.0

[Table/Fig-3]: Diagnostic performance of cytochemistry in ALL and AML.

Morphology	PAS Positive	PAS Negative	MPO Positive	MPO Negative	Morpho cytochemistry diagnosis
Acute leukaemia (n=65)	25	41	13	53	Acute leukaemia (Undifferentiated): 27 AML: 13 ALL: 25
ALL (n=53)	30	23	0	53	ALL: 53
AML (n=51)	2	49	47	4	AML: 51

[Table/Fig-4]: Comparison of morphological diagnosis with PAS and MPO cytochemical staining in acute leukaemia.

Flow-cytometry diagnosis	Morpho cytochemistry diagnosis				Kappa coefficient	p-value
	Acute leukaemia	AML	ALL	Total		
AML	12	63	2	77	0.685#	<0.001
ALL	15	1	76	92		
Total	27	64	78	169		

[Table/Fig-5]: Comparison of flow cytometry with morphocytochemistry diagnosis.
#Cohen's Kappa test

Marker	AML (n=77) n (%)	B-ALL (n=52) n (%)	T-ALL (n=40) n (%)
CD45	77 (100)	50 (96.15)	40 (100)
CD34	50 (64.93)	30 (57.69)	18 (45.00)
CD1a	0	0	23 (57.50)
CD3 (surface)	0	0	17 (42.50)
CD4	20 (25.97)	0	20 (50.00)
CD7	19 (25.67)	0	39 (97.50)
CD8	0	0	23 (57.50)
CD10	0	48 (92.30)	6 (15.00)
CD11b	29/52 (55.77)	2/26 (7.69)	3/22 (13.63)
CD14	8/30 (26.67)	0	0
CD15	32 (41.56)	0	0
CD13	72 (93.50)	10 (19.23)	5 (12.50)
CD16	11 (14.28)	0	2 (5.00)
CD19	7 (9.09)	52 (100)	0
CD33	73 (94.80)	1 (1.92)	6 (15.00)
CD56	10 (12.99)	0	3 (7.50)
CD117	43 (55.84)	0	4 (10.00)
HLA-DR	60 (77.92)	49 (94.23)	2 (5.00)
cMPO	69 (89.61)	0	0
cCD3	0	0	40 (100)
cCD79a	0	52 (100)	0

[Table/Fig-6]: Expression of immunophenotypic markers in acute leukaemia subtypes.
Note: Percentages are calculated using the number of cases tested for each marker. Certain markers were assessed only in subsets of patients.

DISCUSSION

The present study underscores the diagnostic relevance of cytochemistry, morphocytochemistry, and flow cytometry in accurately classifying acute leukaemias and demonstrates how these modalities complement each other within a modern diagnostic framework. PAS continued to show strong specificity for ALL, consistent with the findings of Ooi MG et al., and Arber DA et al., who have similarly reported that PAS positivity remains a reliable indicator of lymphoid lineage, particularly in B-ALL [8,10]. The slightly reduced sensitivity of PAS observed in our cohort may be attributed to the higher prevalence of T-ALL, a subtype that characteristically lacks PAS reactivity, a trend also described in contemporary Indian datasets such as those by Rai V et al., [15]. MPO demonstrated excellent diagnostic accuracy for AML, aligning with recent clinical analyses by Ally F and Chen X and Chaudhary S et al., who highlighted MPO's sustained importance in confirming myeloid lineage, even as molecular assays increasingly refine AML classification [7,14].

Morphocytochemistry successfully classified more than 80% of leukaemia cases, reinforcing its continued clinical applicability. Kumar A et al., similarly demonstrated that morphology combined with cytochemistry remains an effective first-line approach, particularly in low-resource environments where immunophenotyping may not be readily accessible [5]. In this study, morphology alone classified a significantly smaller proportion of cases, but the addition of cytochemical stains markedly improved diagnostic accuracy- an effect also noted by Holl E et al., and Wood BL who underscored the synergistic diagnostic capacity of combining structural and biochemical evaluation [12,13]. Flow cytometry demonstrated the highest diagnostic accuracy, assigning definitive lineage in nearly all cases. The substantial agreement between flow cytometry and morphocytochemistry mirrors findings from Li W and Ooi MG et al., reaffirming that while cytochemistry provides valuable preliminary insights, immunophenotyping remains indispensable for final diagnosis [6,8].

The distribution of leukaemia subtypes in our cohort- especially the relatively higher proportion of T-ALL- aligns with patterns described in tertiary-care-based Indian studies, including Rai V et al., who also reported increased T-ALL prevalence in referral centers [15]. This differs from Western data, such as those by Arber DA et al., where B-ALL predominates, highlighting how regional epidemiology and referral patterns can influence subtype distribution [10]. Similarly, the predominance of AML-M2 and AML-M3 cases in our cohort corresponds with observations in Indian AML datasets summarised by Chaudhary S et al., [14] and supported by updated WHO 2022 and ICC 2022 classification frameworks [2], which document high frequencies of RUNX1-RUNX1T1-positive AML and PML-RARA-associated AML in South Asian populations.

Limitation(s)

The present study has inherent limitations due to its tertiary-care setting, which introduces referral bias and may not reflect population-level subtype prevalence. The absence of additional cytochemical stains such as Sudan black B restricted the characterisation of certain AML subtypes, particularly monocytic variants. Furthermore, the absence of cytogenetic and molecular data precluded the incorporation of genomic markers that currently define several leukaemia subtypes. These constraints underscore the need for future multicentre studies with wider population representation and systematic integration of molecular diagnostic techniques.

CONCLUSION(S)

The present study reaffirms the continued diagnostic significance of cytochemistry, morphocytochemistry, and flow cytometry in the evaluation of acute leukaemias. PAS and MPO remain valuable, highly specific frontline tools, particularly in settings with limited access to advanced technologies. When combined with morphology, cytochemistry markedly improves diagnostic accuracy, highlighting its ongoing relevance in routine haematopathology. Flow cytometry, however, demonstrated superior precision and served as the definitive modality for accurate lineage determination and leukaemia subtyping, showing substantial concordance with morphocytochemistry. The findings underscore the need for an integrated diagnostic approach that incorporates morphology, cytochemistry, and immunophenotyping to achieve optimal classification. Strengthening diagnostic capacity, ensuring access to flow cytometry, and integrating molecular markers in future studies will further refine diagnostic accuracy and enhance patient outcomes in acute leukaemia.

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