Original Article

DOI: 10.7860/NJLM/2023/58267.2706

Pathology Section

Bone Marrow Profile in Haematological Disorders with reference to Flow Cytometry and RT-PCR in Acute Leukaemia

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ABSTRACT

Introduction: Evaluation of non neoplastic and neoplastic haematological disorders require bone marrow examination which is an important diagnostic tool. This includes Bone Marrow Aspiration (BMA) and Bone Marrow Biopsy (BMB). Subtyping of Acute Leukaemia (AL) requires flow cytometry immunophenotyping and Real Time Polymerase Chain Reaction (RT-PCR), which helps in identifying cell antigens and genetic abnormalities, respectively. This is helpful to guide specific treatment for patients.

Aim: To evaluate the clinical profile, cytological and histological pattern of various haematological disorders using bone marrow examination and to determine immunophenotypes using ancillary techniques in patients with AL.

Materials and Methods: This was a cross-sectional observational study conducted in Department of Pathology, Sree Mookambika Institute of Medical Sciences, Kulasekharam, Tamil Nadu, India. Data was collected for a period of two years from May 2020 to April 2022. A total of 62 cases were included. Clinical details and bone marrow examination findings were noted for all BMA and BMB cases that satisfied the inclusion criteria and flow cytometry along with RT-PCR diagnosis was done for suspected AL cases. Analysis was done using Statistical Package for the Social Sciences (SPSS) version 20.0.

Results: Among the 62 cases studied, age of patients ranged from 32 to 81 years. Majority of them were in the 5th to 6th decade. Females 32 (51.6%) were more commonly affected. Pancytopenia 15 (24.2%) was the most common clinical presentation. Total 49 (79%) were diagnosed with BMA and 61 (98.4%) were diagnosed with BMB. Megaloblastic anaemia 16 (25.8%) and acute myeloid leukaemias 6 (9.6%) were the most common cause of benign haematological disorder and haematological malignancy respectively. The RT-PCR test for Break point Cluster-Abelson Tyrosine Kinase (BCR-ABL) and Promyelocytic Leukaemia-Retinoic Acid Receptor Alpha (PML-RARA) fusion gene analysis showed association in patients with Acute Lymphoblastic Leukaemia (ALL) and Acute Myeloid Leukaemia (AML) respectively.

Conclusion: Biopsy, being gold standard, provides details about the pattern, extent of tumour, metastatic deposit and granulomatous pathology, but BMA also proved better for study of the cell. Flow cytometry and RT-PCR were effective tools that enable the identification of immunophenotype in AL as well as to assess treatment progress and predict prognosis.

Keywords: Anaemia, Bone marrow aspiration, Bone marrow biopsy, Cluster of differentiation, Pancytopenia

INTRODUCTION

Haematological disorders are quite frequent in all age group and they range from anaemias, infectious disorders to malignancies. Bone Marrow Examination (BME) has an important role in diagnosis of non neoplastic and neoplastic diseases and assessing patient prognosis [1]. The BME plays a crucial role in bone marrow transplantation. Two important techniques used are BMA and BMB. Both are done simultaneously for accurate evaluation of bone marrow [2]. A BMA is useful in making out better individual cell morphology. It is well suited for examination by cytogenetics, Polymerase Chain Reaction (PCR) and Flow Cytometric methods. Whereas BMB is useful in bone marrow pattern, distribution, inadequate or failed aspiration, focal lesions like granulomatous diseases, bone marrow fibrosis, staging of lymphomas and metastasis [3]. Examination of bone marrow needs clinical details, complete blood count, peripheral blood smear, special stain and immunohistochemistry to diagnose AL, lymphomas and to grade marrow fibrosis in chronic myeloproliferative neoplasms [4].

Acute leukaemia is a highly heterogeneous malignant disease, and it is divided into two major types: AML and ALL. The AML is subclassified into eight subtypes (AML M0-M7) and ALL is subclassified into three subtypes (ALL L1-L3) based on morphology, immunological and molecular genetics. Cytogenetic analysis using World Health Organization (WHO) classification is the gold standard for assessment of AL [5].

The subtyping of AL is an important step for correct treatment and prognosis. Flow cytometry immunophenotyping identifies cell membrane antigens using "Clusters of Differentiation" (CD) markers and fluorochromes that are applied to cell suspension. This also helps to detect minimal residual disease. With many advanced techniques, detection of fusion genes was important for the diagnosis, classification, and prognosis of AL [6]. This study was aimed to evaluate the clinical profile, cytological and histological pattern of various haematological disorders using BME and to determine the immunophenotypes of acute leukaemia using ancillary techniques like flow cytometry and RT-PCR. Objectives of the present study was to diagnose diseases affecting bone marrow using BMA and BMB, to evaluate the expression of immunemarkers in subtyping ALs and to identify BCR ABL and PML RARA fusion using RT-PCR for treatment and prognosis. Flow cytometry and RT-PCR helps to identify immunophenotype of AL and to find the clinical usefulness of these different phenotypes in prognosis of patients with AL.

MATERIALS AND METHODS

This was a cross-sectional observational study carried out in Department of Pathology, Sree Mookambika Institute of Medical Sciences, Kulasekharam, Tamil Nadu, India, conducted over a period of two years from May 2020 to April 2022.

Ethical clearance was obtained (IHEC No:52/2019). Informed consent was obtained from all patients before performing BMA and biopsy.

Based on the study conducted by Fareed N et al., [7], prevalence of hematological disorder was found to be 79.7%.

Sample size calculation: Sample size was calculated using the formula n=Z1-α/22 pq/d2=62.15=62 cases

where, p=79.7%

q=20.3%

d=10% absolute error

Z1-α/2=1.96 for α=5%

Inclusion criteria: All cases undergoing bone marrow studies where both aspiration and biopsy was done.

Exclusion criteria: Cases where only aspiration was available and follow-up cases.

Study Procedure

Clinical details were documented. An Ethylenediaminetetraacetic Acid (EDTA) blood sample was collected from all patients for complete blood count and peripheral smear examination. Smears was prepared for all cases and stained with Leishman stain. The BMA and BMB was done under aseptic precautions from posterior iliac spine. The procedure was done under local anaesthesia (Inj. 2% xylocaine). For aspiration and biopsy, LP 16 G needle and Jamshidi needle were used respectively. Aspiration sample was collected for performing BMA smears, flow cytometry and RT-PCR. A BMA smear was prepared and stained with Giemsa and Perl's stain. The BMB sample was received in Bouins fixative, after grossing it was processed in alcohol and xylene. Embedded in paraffin wax, sections were cut in 3-4 µ thickness, stained with Hematoxylin and eosin stain. Smears and biopsy slides were viewed and diagnosis was reached. In cases of AL, flow cytometry and RT-PCR were done.

For flow cytometry a panel of monoclonal antibodies (Immunotech) were used, that included myeloid markers (MPO, CD13, CD14, CD15, CD33, CD117, and CD11b), T-cell lineage markers (CD2, CD3, cyCD3, CD5, and CD7), B-cell lineage markers (CD10, CD19, and CD20), megakaryocytic lineage markers (CD41 and CD61) and other markers (CD34, CD38, HLA-DR, CD71, CD9,CD25,CD103,CD56, Anti KAPPA and Anti LAMBDA). Comprehensive antibody with direct immunofluorescent labeling was used for immunophenotyping with a FACSCanto II System and Diva software. The blast population was gated on the basis of light scattering properties. Unstained cells in each tube were used as negative controls to set quadrant gates. The BCR ABL and PML RARA fusion gene analysis was done using RT quantitative PCR kit - HG-U133 plus2 (Affymetrix). Each probe included FAM (6-carboxy-fluorescein; emission, 518 nm) at the 5'-end as the reporter and TAMRA (6-carboxy-tetramethylrhodamine; emission, 582 nm) at the 3'-end as the quencher. The primer and probe sequences were designed using Primer Express software.

Non probability sampling technique with all consecutive cases that satisfied the inclusion criteria were included in this study (62 cases). The BMA slides were examined for the cellularity, myeloid erythroid ratio, erythroid series, myeloid series, lymphoid series, plasma cells, histiocytes, megakaryocytes and abnormal cell infiltration. In addition to the findings mentioned in BMA, BMB slides were viewed for pattern of infiltration, granulomas, Abnormal Infiltration of Immature Precursors (ALIP) and marrow fibrosis. In flow cytometry percentage of antigen positive cells (cut off >20%) was calculated using the quadrant statistics. Expression of one or more markers on more than 20% of the blast cell population was considered. Based on the positivity of immune markers subtyping of acute leukaemia was done. The BCR p190 copy number , BCR p210 copy number, ABL copy number and PML RARA copy numbers were assessed and type of transcript was detected in RT-PCR.

STATISTICAL ANALYSIS

Data entered in excel sheet and analysis was done using SPSS 20.0 software. Frequency of haematological disorder was presented as percentage and 95% confidence interval. The association between haematological disorder and demographic variable was tested for statistical significance using Chi-square test. A p-value of less than 0.05 was considered as statistically significant.

RESULTS

A total of 62 cases were included in this study. Patients age ranged from 32 to 81 years. Majority of the cases 15 (24.2%) cases were in 5th to 6th decade. Females 32 (51.6%) were more commonly affected than males 30 (48.4%).

Pancytopenia 15 (24.2%) and anaemia 12 (19.4%) were the most common clinical indications for performing bone marrow examination. Distribution of indications for bone marrow examination is depicted in [Table/Fig-1].

Indications	Frequency	Percent %
Pancytopenia	15	24.2
Anaemia	12	19.4
Pyrexia of Unknown Origin	2	3.2
Splenomegaly	6	9.7
Hepatosplenomegaly	7	10.5
Lymphadenopathy	2	3.2
Suspicious of Multiple Myeloma	9	14.5
Thrombocytosis	1	1.6
Thrombocytopenia	2	3.2
Metastatic work up	6	9.7
Total	62	100

[Table/Fig-1]: Distribution of cases according to indication of bone marrow examination

Association between age , gender and BMB diagnosis was tested and no association was found, since the p-values were 0.3 and 0.4 respectively [Table/Fig-2,3].

Sensitivity specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of BMB was 98,33%, 100%, 100% and 66.66% respectively and in BMA was 76.99%, 100%, 100% and 20% respectively.

In BMB, nutritional anaemia includes both iron deficiency and megaloblastic anaemia (16 cases, 25.8%) was the most common benign haematological disorder and acute leukaemia (12 cases, 19.3%) was the most common malignant haematological disorder in this study [Table/Fig-4]. Among the 62 cases, 49 (79%) were diagnosed with BMAand 98.4%(61 cases) were diagnosed with BMB. Total 61 (98.4%) were diagnosed by both BMA and BMB while 1 (1.62%) was inconclusive because of inadequate sampling technique. The concordance rate and inconclusive cases with final haematological diagnosis (N=62) is described in [Table/Fig-4].

Of the remaining 12 cases that were inconclusive in BMA, 1(1.6%) case was diagnosed as megaloblastic anaemia, 1(1.6%) as iron deficiency anaemia, 1(1.6%) as immune thrombocytopenic purpura and 1(1.6%) as plasma cell dyscrasia [Table/Fig-5,6] in BMB. The BMA in these cases were diluted. About 2 (3.2%) and 4 (6.4%) cases showed myeloproliferative neoplasm [Table/Fig-7,8] and metastatic deposit [Table/Fig-9,10] in BMB respectively. Marrow fibrosis/packed marrow due to infiltration by neoplastic cells can yield hypocellular aspirate. This highlights the importance of trephine biopsy. About 2 (3.2%) cases that had no diagnosis in BMA, showed granulomas in BMB [Table/Fig-11,12]. Fibrosis in and around granuloma may cause difficult marrow aspiration.

Twelve cases of acute leukaemia required other ancillary investigations like flow cytometry and RT-PCR. Flow cytometry was done for subtyping based on WHO classification [6]. Flow cytometry uses different immune phenotypic markers that help to differentiate subtypes of AML and ALL [Table/Fig-13]. Flow cytometry of B ALL

Age (years)	Acute leukaemia	Myeloproliferative Neoplasms	Lymphoproliferative neoplasm	Plasma cell dyscrasia	Iron deficiency anaemia	Megaloblastic anaemia	Metastasis	Myelodysplastic Syndrome	Granulomatous lesion	Normal Study	Immune Thrombocytopenic Purpura	Aplastic anaemia	Inconclusive	Total	p-value
31-40	2	2	0	1	1	7	0	0	0	0	0	0	0	13	
41-50	3	3	0	2	3	0	0	0	1	1	1	0	0	14	
51-60	4	2	1	2	1	0	3	0	0	1	0	1	0	15	
61-70	2	1	0	3	1	0	2	1	1	0	0	1	1	13	0.3
71-80	1	0	0	0	1	2	1	1	0	0	0	0	0	6	
81-90	0	0	0	1	0	0	0	0	0	0	0	0	0	1	
Total	12	8	1	9	7	9	6	2	2	2	1	2	1	62	

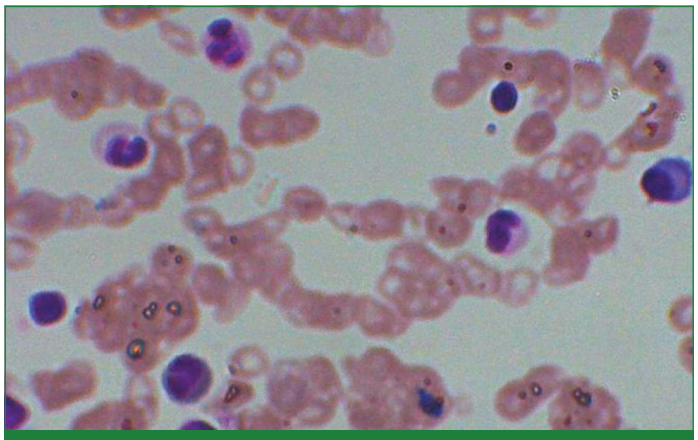
[Table/Fig-2]: BMB diagnosis based on age distribution.

Parameters	Acute leukaemia	Myeloproliferative Neoplasms	Lymphoproliferative neoplasm	Plasma cell dyscrasia	Iron Deficiency anemia	Megaloblastic anemia	Metastasis	Myelodysplastic syndrome	Granulomatous lesion	Normal study	Immune thrombocytopenic purpura	Aplastic anemia	inconclusive	Total	p-value
Male	4	4	1	3	1	6	3	1	2	1	1	2	1	30	
Female	8	4	0	6	6	3	3	1	0	1	0	0	0	32	0.4
Total	12	8	1	9	7	9	6	2	2	2	1	2	1	62	

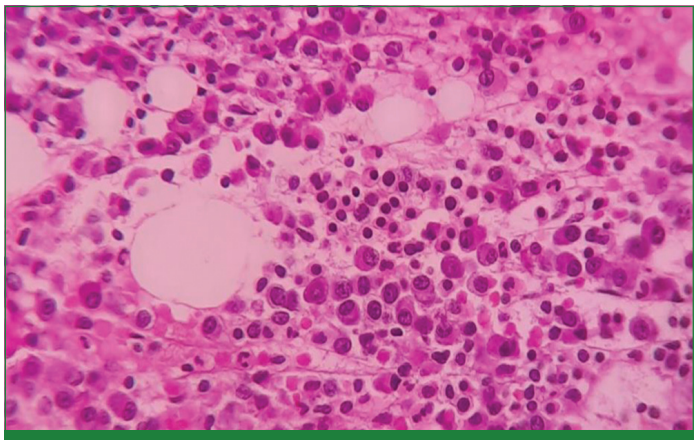
[Table/Fig-3]: BMB diagnosis based on gender distribution.

Diagnosis	BMB		BMA		BMB & BMA (concordance)	Inconclusive in BMA but diagnosed with BMB	Inconclusive BMA & BMB
	Frequency	%	Frequency	%			
Acute leukaemia	12	19.3	12	19.3	12 (100%)	0	0
Myeloproliferative Neoplasms	8	12.9	6	9.7	6 (75%)	2 (25%)	0
Lymphoproliferative neoplasm	1	1.6	1	1.6	1 (100%)	0	0
Plasma cell dyscrasia	9	14.5	8	12.9	8 (89%)	1 (11%)	0
Iron deficiency anaemia	7	11.3	6	9.7	6 (86%)	1 (14%)	0
Megaloblastic anaemia	9	14.5	8	12.9	8 (89%)	1 (11%)	0
Metastasis	6	9.7	2	3.2	2 (33%)	4 (67%)	0
Myelodysplastic syndrome	2	3.2	2	3.2	2 (100%)	0	0
Granulomatous lesion	2	3.2	0	0	0	2 (100%)	0
Normal marrow study	2	3.2	2	3.2	2 (100%)	0	0
Immune thrombocytopenic purpura	1	1.6	0	0	0	1 (100%)	0
Aplastic anaemia	2	3.2	2	3.2	2 (100%)	0	0
Inconclusive	1	1.6	13	21.1	0	0	1 (100%)
Total	62	100	62	100	49	12	1

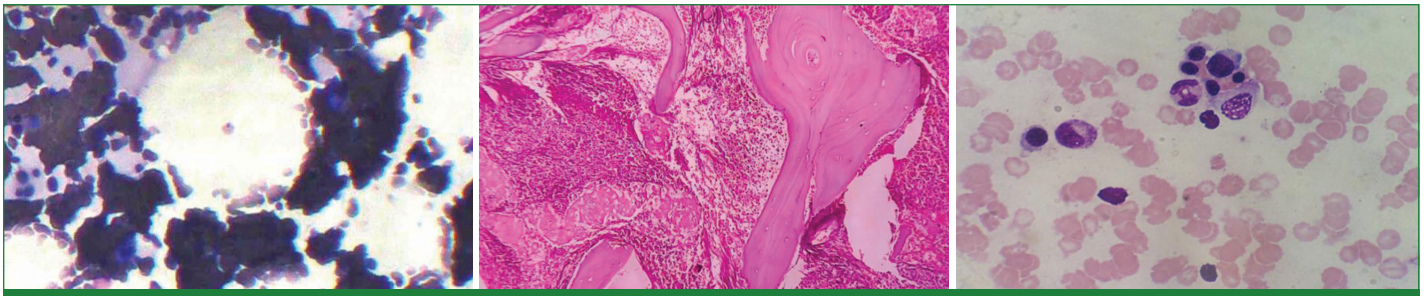
[Table/Fig-4]: Comparison of BMB and BMA diagnosis of haematological disorders.



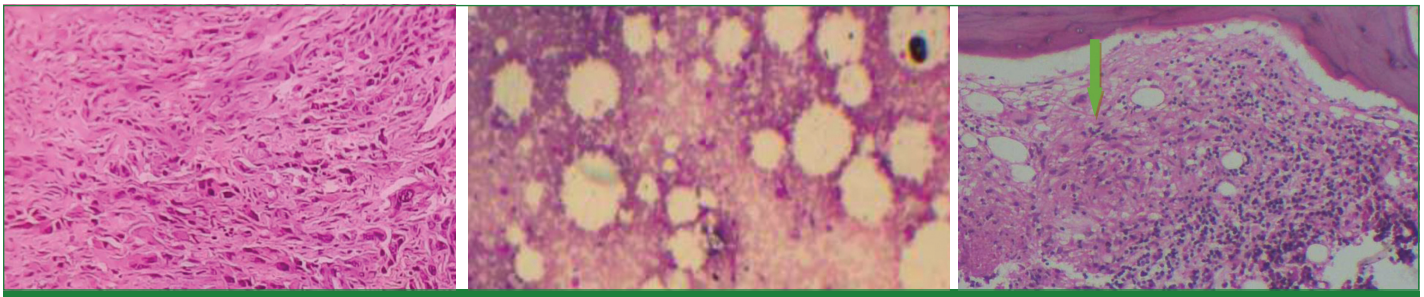
[Table/Fig-5]: Case-1: Diluted BMA with rare plasma cell (Leishman stain 40X).



[Table/Fig-6]: Case-1: BMB plasma cell dyscrasia – multiple myeloma (H&E 40X).



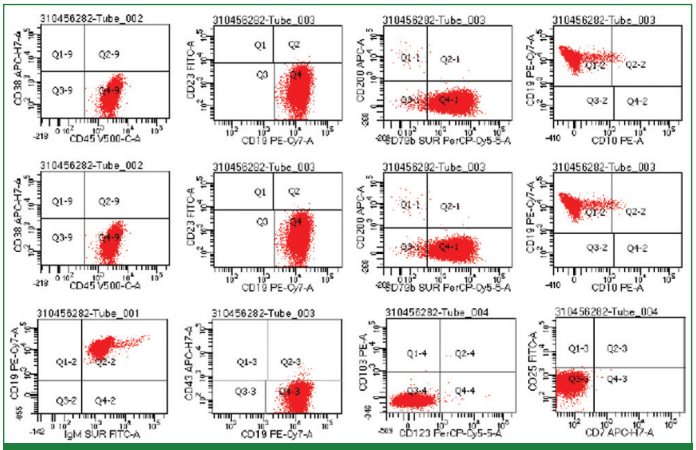
[Table/Fig-7]: Case-2: Diluted BMA showing few erythrpid cells (Leishman stain 10X). **[Table/Fig-8]:** Case-2: BMB with chronic myeloid leukaemia showing marrow firbrosis (H&E 4X). **[Table/Fig-9]:** Case-3: Case-3: Diluted BMA with occasional erythroid and myeloid precursor cells (Leishman stain 40X). (Images from left to right)



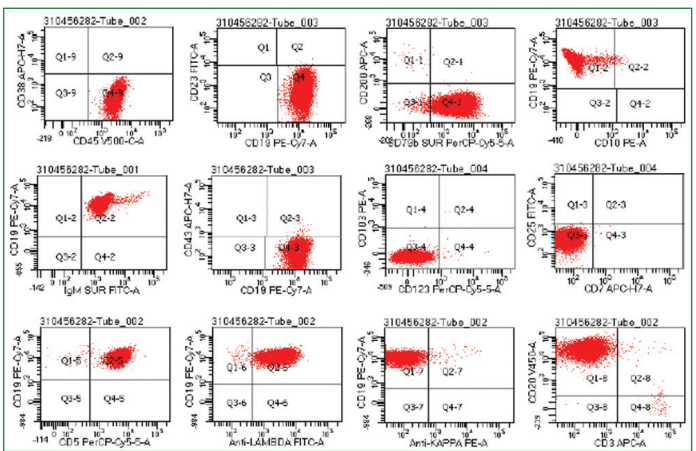
[Table/Fig-10]: BMB with metastatic tumour cell deposits (H&E 10X). **[Table/Fig-11]:** Case-4: Diluted BMA with occasional erythroid precursor cells (Leishman stain 4X). **[Table/Fig-12]:** Case-4: BMB with granuloma (H&E 10X) (arrow). (Images from left to right)

Flow cytometry diagnosis	Frequency
Precursor B-ALL	4
Precursor T-ALL	1
AML M2	2
AML M3	3
AML M4	1
Leukemic phase of mantle cell lymphoma	1

[Table/Fig-13]: Flow cytometry diagnosis of acute leukaemias.



[Table/Fig-14]: Flow cytometry for precursor B-ALL.



[Table/Fig-15]: Flow cytometry for leukaemic phase of mantle cell lymphoma.

and leukemic phase of mantle cell lymphoma was shown in [Table/Fig-14,15] respectively.

The RT-PCR was done in acute leukaemia cases (12 cases) to look for BCR and PML fusion gene cytogenetics [Table/Fig-16].

	BCR p190		BCR p210		PML	
Variables	+	-	+	-	+	-
B ALL (n=4)	3	1	1	3	0	4
T ALL (n=1)	1	0	0	1	0	1
AML M2 (n=2)	0	2	0	2	0	2
AML M3 (n=3)	0	3	0	3	3	0
AML M4 (n=1)	0	1	0	1	0	1
Leukemic phase of mantle cell lymphoma (n=1)	0	1	0	1	0	1

[Table/Fig-16]: RT-PCR for acute leukaemia cases.

DISCUSSION

In this study the patient were in age group between 31- 60 years. This is similar to study conducted by Fareed N et al., [7]. There is a wide range of haematological disorders. Bone marrow examination is easily available in many of the hospitals and is one of the important diagnostic technique. It is a combination of clinical assessment from history and examination of patient and different staining preparation on BMA and trephine slides [8].

In this study, females 32 (51.6%) were more commonly affected than males 30 (48.4%). Contrary to present study, Male preponderance was observed by Joshi-Warpe S et al., [9] with a male to female ratio of 1.27:1. Fareed N et al., [7] in their study showed 27 (47.4%) were males and 30 (52.6%) were females which is similar to this study. The commonest indication of bone marrow examination was pancytopenia. This is similar to studies done by Ranabhat S et al., [10]. Study conducted by Bashir N et al., reported incidence of pancytopenia in 54.5% cases [11].

Among anaemias, megaloblastic anaemia was more common 9 (14.5%) followed by iron deficiency anaemia 7(11.3%) in BMB. The BMA smears were stained with Perl's stain for grading iron stores. Bashir N et al., [11] in their study showed that megalobaalstic anaemia was the most common type. Alwahaibi NY et al., showed that BMA smears were better for examining iron stores when compared to BMB specimens that were decalcified with formic acid in diagnosing iron deficiency anaemias that is similar to our study [12].

Acute leukaemia was diagnosed in 12 cases (19.3%), AML was the most common type. Ranabhat S et al., [10] and Vijayamohanan L et al., [13] also showed similar findings. All AL cases were diagnosed in bone marrow aspiration. Trephine biopsy sections render information which cannot be determined from aspiration, such as spatial distribution and extent of infiltrates and overall cellularity. This is comparable to study done by Puri V et al., [14]. Among the 9 (14.5%) cases of plasma call dyscrasias, 7(11.6%) cases were of multiple myeloma that was comparable to study done by Reshma ST et al., [15] who reported that incidence of plasma cell dyscarsia was 9.57%.

The ITP was diagnosed in 1 case (1.6%) in this study which was comparable to study conducted by Bashir N et al., [11] and Reshma ST et al., [15] who reported 1.3% and 0.87% respectively. Total 8 (12.9%) cases were diagnosed as myeloproliferative neoplasms in BMB. In CML cases, aspirates were better able to classify the phases as compared to biopsy. Two cases of granulomatous pathology were diagnosed on BMB. Aspirate showed reactive marrow similar to study done by Gilotra M et al., [16]. About 49 (79%) and 61 (98.4%) cases in this study were diagnosed with BMA and biopsy respectively. Gilotra M et al., [16] in their study diagnosed 87% cases with BMA and the remaining required biopsy for correct diagnosis. Total 49 (80.33%) cases showed concordance in both BMA and BMB, and was comparable to study conducted by Gilotra M et al., [16] with 72.4% concordance in BMA and BMB.

In this study sensitivity for diagnosing haematological disorders was high for BMB (98.33%) when compared to BMA (76.99%), so BMB was considered as gold standard. This is similar to Goyal S et al., [17] who showed a sensitivity of 88.5%. Among the 12 AL cases diagnosed on BMA and biopsy, flow cytometry found 4(6.4%) B-ALL, 1 (1.6%)T-ALL, 2 (3.2%) AML M2, 3(4.8%) AML M3, 1(1.6%) AML M4 and 1 (1.6%) leukemic phase of mantle cell lymphoma. Large number of cells can be examined quickly by flow cytometry, which improves the accuracy of leukaemia diagnosis and helps in detection of mixed phenotypic AL [18].

In the present study, all patients with AML M3 were BCR-ABL p190 and p210 negative, whereas four patients with ALL were BCR-ABL p190 positive and p210. The AML-M3 cases showed PML positivity. A study revealed that patients of AL who exhibit BCR-ABL mutations seemed to have a worse prognosis than those without these mutations [19]. This states that detection of fusion gene was important for the diagnosis, classification, and prognosis of AL [20].

Limitation(s)

Touch imprints were not evaluated in study which may have increased the diagnostic accuracy. Analysis of cytogenetic immunophenotype correlations was not performed due to the small sample size in this study. A larger sample size is needed to explore this association in future studies.

CONCLUSION(S)

This study concluded that BMA and BMB were complement to each other and the superiority of one method over the other depended on the underlying disorder. Both procedures are used routinely nowadays and do not require sophisticated equipment's. Hence BMA and BMB should be done simultaneously as they play important role for making diagnosis of haematological disorders. Further, flow cytometry and RT-PCR analysis provides rapid, and effective method to aid in diagnosis, treatment prognosis and to predict prognosis in patients with AL.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]

- Plagiarism X-checker: Jun 16, 2022
- Manual Googling: Aug 20, 2022
- iThenticate Software: Sep 10, 2022 (19%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:

- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. Yes

Date of Submission: **Jun 07, 2022**
Date of Peer Review: **Jul 22, 2022**
Date of Acceptance: **Sep 12, 2022**
Date of Publishing: **Jan 01, 2023**