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Original Article

Pathology Section

Multiparameter Flowcytometry for Diagnosis and Subtyping of Mature Lymphocytic Neoplasms in Peripheral Blood and Bone Marrow: A Prospective Observational Study

SHAILENDRA JAMBHULKAR[1], PURNIMA KODATE[2], MANJIRI MAKDE[3], MUKESH WAGHMARE[4], JAYSHRI TIJARE[5], DT KUMBHALKAR[6]

ABSTRACT

Introduction: Multiparameter Flowcytometry (MFC) is a high throughput, quick, and practical technique for diagnoses of Chronic Lymphoproliferative Disorders (CLPDs). Indian CLPDs cases have distinct distribution and presentation than the developed world. Moreover, limited studies have confirmed the diagnostic utility of MFC in Indian CLPDs cases.

Aim: To evaluate the diagnostic utility of MFC in peripheral blood and bone marrow aspirate of CLPDs cases.

Materials and Methods: This was a single-centre, prospective, observational study involving clinico-morphologically suspected or diagnosed 85 CLPDs cases. It was carried out in the Department of Pathology, Government Medical College and Hospital, Nagpur, Maharashtra, India, from January 2016 to November 2019. The patients were followed-up for peripheral smear (PS) and Bone Marrow (BM) MFC and staging in nodal or extranodal Non-Hodgkin's Lymphoma was done.

Results: Clinico-morphological examination led to the diagnosis of 74 CLPD cases, while remaining 11 cases were strongly suspected. MFC immunophenotyping was contributory in diagnosing 74 CLPD cases which on further subtyping consisted of B-cell CLPD (N=70), and T-cell CLPD (N=3), while one case of B-NHL could not be subtyped. The most common B-cell CLPD included multiple myeloma (n=27), chronic lymphocytic leukaemia (n=25), diffuse large B-cell lymphoma (n=7). T-cell CLPD included hepatosplenic gamma delta T-cell lymphoma (N=2) and adult T-cell lymphoma, follicular lymphoma (n=3), burkitt's lymphoma (n=2), mantle cell lymphoma (n=2), pro-lymphocytic leukaemia (n=2), splenic marginal zonal lymphoma (n=2), B-Cell Non-Hodgkin's Lymphoma (n=1). Finally, 11 suspected cases mostly comprised of reactive lymphocytosis (81.8%).

Conclusion: The MFC immunophenotyping led to diagnoses and determination of CLPD sub-class. It also resulted in rapid diagnoses of reactive hyperplasia and non haematolymphoid malignancy which may mimic CLPD on morphology and hence, difficult to diagnose based on morphology alone.

Keywords: Immunophenotyping, Leukaemia, Lymphoma, Multiple myeloma

INTRODUCTION

Lymphoid neoplasms are a diverse group of malignant clonal tumours of mature and immature B-Cells, T cells, or NK cells at different stages of differentiation [1]. Neoplasms arising from mature lymphoid cells include lymphoid neoplasms, chronic leukaemias, and non-Hodgkin lymphomas. They are distinguished by an immunophenotype that is identical to normal mature lymphoid cells (e.g., surface immunoglobulin on mature B-Cells) and absence of antigenic characteristics of immature cells (including expression of TdT, CD34, or weak intensity staining for CD45) [2].

Flow Cytometric (FCM) studies involve evaluation of physical properties and patterns of antigen expression in a solution of undamaged cells, and provide diagnostic immunophenotypic profile in majority of the cases with abnormal cells in the blood or Bone Marrow (BM) aspirate [3,4]. Together with molecular and cytogenetics analysis, it is a vital modality for diagnoses and classification of diseases such as leukaemias and lymphomas [5]. Moreover, it is used for assessment of tumour cell DNA and cell cycle analysis [6].

Subsequently, Multiparameter Flowcytometric (MFC) immunophenotyping was introduced. Because of its high throughput, quick availability of results, and practicable logistics, it has become a dominant technique for demonstrating the ontogeny in B-cell lymphoproliferative disorders (LPD). Use of MFC for Immunophenotyping of Chronic LPD (CLPDs) or mature lymphocytic neoplasms results in quick and comprehensive antigen expression profile which leads to a definitive diagnosis in majority of cases or atleast aid in narrowing the differentials. Precise and timely diagnosis of CLPDs is vital for starting early and appropriate clinical treatment [7].

However, with respect to Indian population, limited studies have confirmed the diagnostic utility of MFC in patients with mature lymphocytic neoplasm [8]. Hence, this study was planned to evaluate the diagnostic utility of MFC in patients with CLPDs in Peripheral Blood (PB) and BM.

MATERIALS AND METHODS

This was a single-centre, prospective, observational study involving clinico-morphologically suspected or diagnosed 85 CLPDs cases. It was carried out in the Department of Pathology, Government Medical College and Hospital, Nagpur, Maharashtra, India, from January 2016 to November 2019. The approval of Institutional Ethics Committee and written informed consent of patients were obtained prior to initiation of the study.

Inclusion criteria: Patients of any age group, either sex, with clinico-morphologically suspected or diagnosed cases of mature lymphocytic neoplasms or CLPDs, unexplained persistent lymphocytosis, and requiring Peripheral Smear (PS) and BM MFC for staging in nodal or extranodal Non-Hodgkin's Lymphoma were included in the study.

Exclusion criteria: The patients with acute lymphoblastic leukaemia, acute myeloblastic leukaemia, chronic myeloid leukaemia, and reactive viral lymphocytosis were excluded.

Study Procedure

The patients fulfilling the eligibility criteria were followed-up, and PS and BM samples were obtained after explaining the procedure. The sample collection procedure helped to acquire material for further immunophenotyping by MFC. In clinico-morphologically suspected or diagnosed cases of CLPD, complete blood count, and PS and BM smears were examined as an initial investigation, to rule out acute leukaemia. Smears were air dried and stained with Leishman's method and examined under light microscopy for the presence of atypical lymphoid cells. Subsequently, the suspicious or diagnosed cases were advised PB collection (from antecubital vein) and BM aspiration (from posterior superior iliac spine), following which samples were collected in Ethylene Diamine Tetra-acetic Acid (EDTA) bulb for MFC.

Immunophenotyping: To perform MFC immunophenotyping (MFCI), a repeat PB collection or BM aspiration was done. Single cell suspension was obtained by lyse stain and wash method, and adequacy of the suspension was counted by loading suspension drop on improved Neubauer's chamber with the cell counts adjusted to approximately 10,000 cells/μL, by concentration after centrifugation or diluting with Phosphate Buffer Saline (PBS) [9].

Preparation of PBS: Sodium chloride (40 g), disodium hydrogen phosphate (13 g), albumin (10 g), potassium chloride (10 g), potassium dihydrogen phosphate (1 g), and sodium azide (1 g) were added in 1 litre of distilled water. The working (1× buffer) solution was prepared when needed. The pH was checked every day with pH paper and maintained between 7.0 and 7.5.

Preparation of erythrocyte lysing reagent (RBC lyse): The reagent was prepared by mixing Ammonium chloride (41.3 g), Potassium bicarbonate (5 g), and tetra Sodium EDTA (0.185 g) in 1 litre of distilled water. The working 1× lysing reagent was prepared when needed. The pH was checked every day with pH paper and maintained at 7.2.

Procedure for preparation of single cell suspension (lyse stain wash): The Red Blood Cell (RBC) lysing reagent (4 mL) was added to 400 μL of sample aspirated in a falcon tube and mixed. The suspension was kept for 15 minutes at room temperature. It was then centrifuged for 2 min at 540 g and the supernatant was discarded by inverting the tube or using a pipette without disturbing the pellet. The pellet was broken and PBS (3 mL) was added and mixed following which it was centrifuged for 2 min at 540 g and the supernatant was discarded by inverting the tube or using a pipette without disturbing the pellet. The above wash procedure with PBS was repeated once again and the supernatant was discarded without disturbing the pellet. Finally, PBS (300 μL) was added to the pellet to adjust the cell counts such that yield was 0.5-1 million cells per 50 μL. Adequacy of the neoplastic cells was evaluated by counting the number of cells after loading a drop of suspension in improved Neubauer's chamber.

Procedure for staining surface antigens: Falcon tubes were labelled as patient's identity (ID), the case requirement (B-tube, T-tube, and Additional tube, if required), and 50 μL of cell suspension was pipetted out into the tube and mixed gently. The required cell surface antibody cocktails (50 μL) was added as determined by titration into the tube. Tubes were incubated for 20 min at room temperature in dark. PBS (2 mL) was again added and mixed gently and centrifuged for 2 min at 540 g and supernatant was discarded. Finally, cell pellet was re-suspended in PBS (300 μL) and ready to be acquired by MFC.

Procedure for intracellular staining: Falcon tubes were labelled for cytoplasmic staining and allotted tube numbers and 50 μL of cell suspension was pipette out into the tube and mixed gently, CD45 antibody was added to it and surface staining procedure was performed as per above steps. Then 100 μL of reagent 1 (IntraPrep fixation, B61411AA, Beckman Coulter) was added and incubated for 15 min at room temperature in dark. PBS (2 mL) was added, mixed well, and centrifuged for 2 min at 540 g and the supernatant was discarded without disturbing the pellet. Pellet was tapped gently and broken, and then 100 μL of reagent 2 (IntraPrep Permeabilisation reagent, B61412 AA, Beckman Coulter) was added with appropriate volume of intracellular antibodies as per the cases. Tubes were incubated for 15 min at room temperature in dark and PBS (2 mL) was again added, mixed gently, and centrifuged for 2 min at 540 g and the supernatant was discarded. Finally, the cell pellet was re-suspended in PBS (300 μL) and was then acquired by MFC.

Fluorochromes: Pacific blue (PB), Fluorescein isothiocyanate (FITC), Phycoerythrin (PE), PE-Texas red (ECD), PE-cyanine 5.5 (PE-Cy5.5), PE-Cyanine 7 (PE-Cy7), Allophycocyanin (APC), APC Alexa fluor 700 (APC AF700), and APC Alexa fluor 750 (APC AF750).

Markers: [Table/Fig-1] depicts the panel of markers used to diagnose and sub-classify the CLPD. One unstained tube was run along the following panel to check autofluorescence of cells.

Tube no.	1	2	3	4	5	6	7	8	9
Fluoro-chrome	PB	FITC	PE	ECD	PC5.5	PC7	APC	APC700	APC750
Backbone panel									
B-tube	-	Kappa	Lambda	-	CD19	CD10	CD5	CD45	CD20
Clone	-	Polyclonal	Polyclonal	-	J3.119	ALB1	BL1a	J.33	LS198.43
T-tube	CD3	CD4	CD7	CD34	-	-	CD22	CD45	CD8
Clone	UCHT1	13B8.2	8H8.1	581	-	-	SJ10.1H11	J.33	B9.11
Additional tube (CD5 positive CLPD/B-NHL cases)									
Add.1*	-	CD43	CD200	-	CD19	-	CD23	CD45	CD38
Clone	-	DFT1	OX-104	-	J3.119	-	9P25	J.33	HP2/1
Additional tube (CD5 negative and CD 10 negative CLPD/B-NHL cases)									
Add.2*	-	CD103	CD11c	CD123	CD19	CD25	CD22	CD45	CD20
Clone	-	2G5	BU-15	SSDC, LY107, D2	J3.119	B1.49.9	SJ10.1H11	J.33	B9E9HRC20
Additional tube (Hodgkin's lymphoma/ALCL cases)									
Add.3*	-	CD15	CD90	CD3	CD19	CD25	CD22	CD45	CD20
Clone	-	80H5	F15-42-1-5	UCHT1	J3.119	B1.49. 9	SJ10.1H 11	J.33	B9E9HRC20
Additional tube (myeloid sarcoma/blast like features)									
Add.4*	-	-	CD13	CD34	CD4	CD117	CD33	CD45	CD38
Clone	-	-	SJ1D1	581	13B8.2	104D2D1	D3HL60.25	J.33	LS198.4.3

[Table/Fig-1]: Panel of markers used to diagnose and sub-classify the CLPD.
PB: Pacific blue; FITC: Fluorescein isothiocyanate; PE: Phycoerythrin ECD: PE-Texas red PC5.5: PE-cyanine 5.5; PC7: PE-Cyanine 7; APC: Allophycocyanin; APC700: APC Alexa fluor 700 ; APC750: APC Alexa fluor 750; CD: cluster of differentiation; *selective markers from additional tubes were added as per respective case requirement

Sample acquisition and analysis: The sample acquisition was done by 3 laser and 10 colour Navios Beckman Coulter flow cytometer by collecting 50,000 ungated list mode events and the MFC data was analysed using Kaluza software version 1.3.

The analysis was done as per the templates that followed fixed gating and dot plot sequences such as: a dot plot with "Time gate" was used for monitoring the acquisition process; a dot plot of "Singlet gate" was first used to exclude the doublet events; CD45 versus side scatter (SSC) dot plot was used to define different cell populations in the sample and also used as gating strategy to isolate specific population; and the gated suspicious cells were further analysed for the antigen expression which were interpreted as positive (as per comparison with the intensity of normal T-lymphocytes; bright, moderate, dim, variable, and subset) and negative.

Data quality assurance: To check the quality control of MFC, the commercially calibrated flow check beads were used with photomultiplier tube, stability, sensitivity, and compensation settings. Flow-check Pro was run on a daily basis and the mean channel fluorescence intensity from each of the measured parameters was established in the Levy Jennings plots. Failure of the peaks to fall within the target regions indicated an instrument problem, which were investigated as and when required. The product used to assess laser alignment and the primary statistical parameter used was Half Peak Coefficient of Variation (HPCV) and was within the following limits: <2% on the Forward scatter (FS), Fluorochrome 1 (FL1), Fluorochrome 2 (FL2), Fluorochrome 3 (FL3), and Fluorochrome 4 (FL4); <2.54% on Fluorochrome 5 (FL5); and < 3% on Fluorochrome 6 (FL6), Fluorochrome 7 (FL7), and Fluorochrome 8 (FL8).

In case of multiple myeloma (MM) patient, examination of urine Bence Jones protein and serum M band examination was done by agarose gel electrophoresis.

STATISTICAL ANALYSIS

This study was descriptive in nature. Parametric and non parametric data were represented in terms of mean±standard deviation and frequencies (percentages), respectively.

RESULTS

On clinico-morphological examination, of 85 cases, 74 were diagnosed as CLPD and 11 were strongly suspected. On MFC, of 74 cases, 73 were confirmed and 1 was false positive. Moreover, of all 11 cases strongly suspicious of CLPD, 1 each was found to be small lymphocytic lymphoma (SLL) and T-cell acute lymphoblastic leukaemia (T-ALL); while, others were reactive (n=9). Of the confirmed cases, the majority were diagnosed with MM (36.5%) followed by chronic lymphocytic leukaemia (CLL, 33.8%). While, of the discordant cases, the majority were diagnosed with reactive lymphocytosis (81.8%) [Table/Fig-2].

Diagnosis (N=85)	n (%)
A. Confirmed cases (N=74)	
a. B-cell CLPDs (n=71)	
Multiple myeloma (MM)	27 (36.5%)
Chronic lymphocytic leukaemia (CLL)	25 (33.8%)
Diffuse large B-cell lymphoma (DLBCL)	7 (9.4%)
Follicular lymphoma (FL)	3 (4.1%)
Burkitt's lymphoma (BL)	2 (2.7%)
Mantle cell lymphoma (MCL)	2 (2.7%)
Pro-lymphocytic leukaemia (PLL)	2 (2.7%)
Splenic marginal zonal lymphoma (SMZL)	2 (2.7%)
B-cell Non-Hodgkin's Lymphoma (B-NHL)	1 (1.6%)
b. T-cell CLPDs (n=3)	
Hepatosplenic gamma delta T cell lymphoma (HSTL-γ,δ)	2 (2.7%)
Adult T-cell lymphoma (ATLL)	1 (1.6%)
B. Suspected cases (N=11)	
Reactive lymphocytosis	9 (81.8%)
Small lymphocytic lymphoma (SLL)	1 (9.1%)
T-cell acute lymphoblastic leukaemia (T-ALL)	1 (9.1%)

[Table/Fig-2]: Distribution of all study subjects based on MFC immunophenotyping.

The mean age of 74 cases diagnosed as CLPD was 57.3±12.7 years. The majority of the cases were males (58.1%) and the male-to-female ratio was 1.4:1. Cases predominantly belonged to the age group of 61–70 years (n=28). Distribution of cases according to clinical presentation revealed that 55 cases had fever, 59 had weight loss, 20 had hepatosplenomegaly, 24 had splenomegaly, and 37 had lymphadenopathy. Of 27 cases with MM, the majority had osteolytic lesion on spine (81.5%), followed by skull (59.3%), ribs (29.6%), and pelvis (18.5%). Moreover, of 27 cases with MM, 21 had M-band in serum and 6 had BJ proteins in urine.

The analysis of CD45 expression combined with SSC in MFC provided a simple method for distinguishing cell lineages. Of 74 CLPD cases, 52 presented with CD45+ and predominantly low side scatter. Among these 52 cases, 27 were moderately positive, 17 were brightly positive, and 8 were dim positive. Of 27 cases of MM, 5 were CD45+ [Table/Fig-3].

Diagnosis	N=52	%
Adult T-cell lymphoma/leukaemia (N=1)	1	100
Burkitt's lymphoma (N=2)	2	100
Chronic lymphocytic leukaemia (N=25)	25	100
Diffuse large B-cell lymphoma (N=7)	7	100
Follicular lymphoma (N=3)	3	100
Hepatosplenic gamma delta T cell lymphoma (N=2)	2	100
Mantle cell lymphoma (N=2)	2	100
Multiple myeloma (N=27)	5	18.5
Pro-lymphocytic leukaemia (N=2)	2	100
Splenic marginal zonal lymphoma (N=2)	2	100
B-Cell Non-Hodgkin's Lymphoma (N=1)	1	100

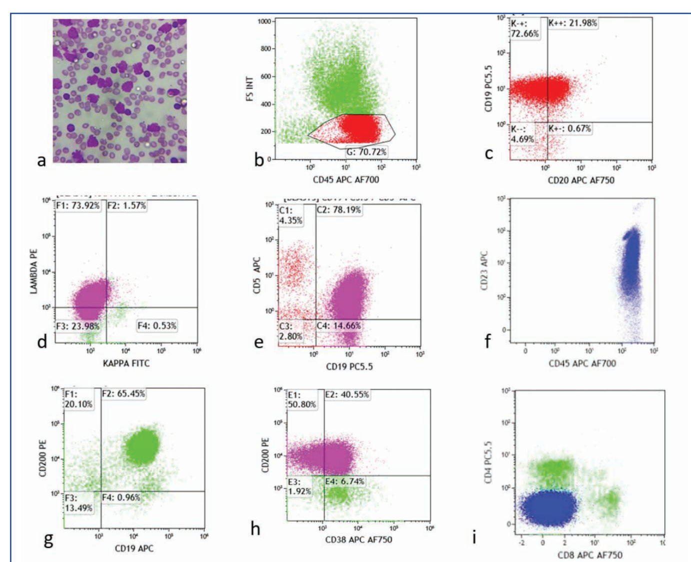
[Table/Fig-3]: Expression of CD45 on MFC immunophenotyping.

The B-tube backbone panel was used to diagnose and sub-classify B-CLPD cases (71 cases) and rule out T-CLPD cases. Of 74 CLPD cases, surface kappa (κ) restriction was seen in 49 and surface lambda (λ) restriction was seen in 22. While, 3 cases were T-CLPD with both κ and λ negative. CD19, CD20, and CD22 were specific to diagnose B-Cell CLPDs, as they were positive in majority cases except MM (n=25), hepatosplenic gamma delta T cell lymphoma (HSTL-γ,δ, n=2), and ATLL (n=1). CD5+ was observed in 29 cases consisting of CLL (n=25), mantle cell lymphoma (MCL, n=2), MM (n=1), and adult T-cell lymphoma (ATLL, n=1). CD10+ was observed in 9 cases including diffuse large B-cell lymphoma (DLBCL, n=4), follicular lymphoma (FL, n=3), and Burkitt's lymphoma (BL, n=2). CD23+ was observed in 29 cases including CLL (N=23) and MM (n=6). CD43 positivity was observed in 41 cases including CLL (n=18), MM (n=13), DLBCL (n=3), BL (n=2), pro-lymphocytic leukaemia (PLL, n=2), ATLL (n=1), MCL (n=1), and splenic marginal zonal lymphoma (SMZL, n=1). CD200+ was observed in 38 cases including CLL (n=25), MM (n=7), FL (n=3), SMZL (n=2), and DLBCL (n=1). CD38+ was observed in 58 cases including MM (N=27), CLL (n=16), DLBCL (n=6), BL (n=2), MCL (n=2), PLL (n=2), ATLL (n=1), FL (n=1), and SMZL (n=1). Finally, CD138+ was observed in all cases of MM (n=27) [Table/Fig-4-6] illustrates the MFC of CLL and MM.

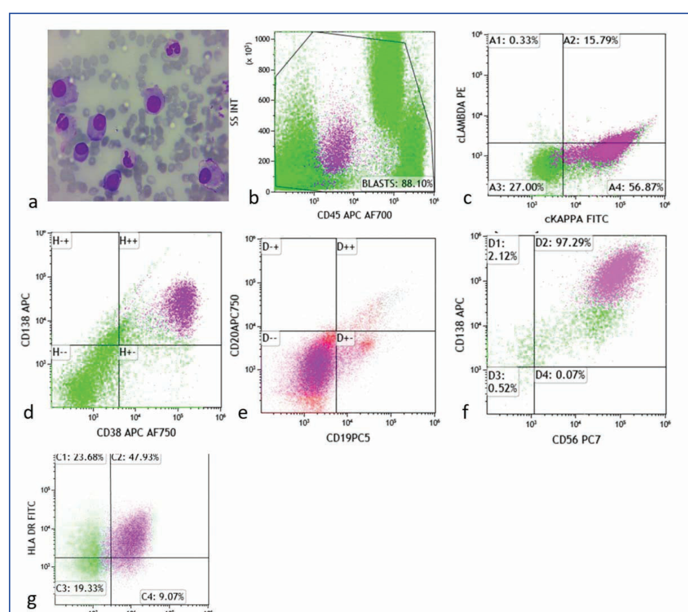
Immunophenotyping (MFCI) of PB and BM aspirates was useful in detecting T-cell CLPDs cases [ATLL (n=1) and HSTL-γ,δ (n=2)] by loss or aberrant (dim or variable) expression of the pan-T-cell antigen like CD3, CD5, and/or CD7 and CD4/CD8- in HSTL-γ,δ and CD4/CD8+

Diagnosis		CD markers											
		CD19+	CD20+	CD 22+	CD5+	CD 10+	CD 23+	CD 43+	CD 200+	CD 38+	CD 138+	sKappa	sLambda
ATLL (N=1)	N	0	0	0	1	0	0	1	0	1	0	0	0
	%	0	0	0	100	0	0	100	0	100	0	00	00
BL (N=2)	N	2	2	2	0	2	0	2	0	2	0	2	0
	%	100	100	100	0	100	0	100	0	100	0	100	0
CLL (N=25)	N	25	25	25	25	0	23	18	25	16	0	13	12
	%	100	100	100	100	00	92	72	100	64	0	52	48
DLBCL (N=7)	N	7	7	7	0	4	0	3	1	6	0	5	2
	%	100	100	100	0	57.1	0	42.8	14.3	85.7	0	71.4	28.6
FL (N=3)	N	3	3	3	0	3	0	0	3	1	0	3	0
	%	100	100	100	0	100	0	0	100	33.3	0	100	0
HSTL-γ,δ (N=2)	N	0	0	0	0	0	0	0	0	0	0	0	0
	%	0	0	0	0	0	0	0	0	0	0	0	0
MCL (N=2)	N	2	2	2	2	0	0	1	0	2	0	2	0
	%	100	100	100	100	0	0	50	0	100	0	100	0
MM (N=27)	N	1	2	2	1	0	6	13	7	27	27	19	8
	%	3.7	7.4	7.4	3.7	0	22.2	48.1	25.9	100	100	70.4	29.6
PLL (N=2)	N	2	2	2	0	0	0	2	0	2	0	2	0
	%	100	100	100	0	0	0	100	00	100	0	100	0
SMZL (N=2)	N	2	2	2	0	0	0	1	2	1	0	2	0
	%	100	100	100	0	0	0	50	100	50	0	100	0
B-NHL (N=1)	N	1	1	1	0	0	0	0	0	0	0	1	0
	%	100	100	100	0	0	0	0	0	0	0	100	0
Total		45	46	46	29	9	29	41	38	58	27	49	22

[Table/Fig-4]: Expression of B-tube CD markers in cases diagnosed with MFC immunophenotyping.
ATLL: Adult T-cell lymphoma; BL: Burkitt's lymphoma; B-NHL: B-Cell Non-Hodgkin's Lymphoma; CLL: Chronic lymphocytic leukaemia; DLBCL: Diffuse large B-cell lymphoma; FL: Follicular lymphoma; HSTL gamma, delta: Hepatosplenic gamma delta T cell lymphoma; MCL: Mantle cell lymphoma; MM: Multiple myeloma; PLL: Pro-lymphocytic leukaemia; SMZL: Splenic marginal zonal lymphoma



[Table/Fig-5]: Chronic lymphocytic leukaemia. PS: cells are small with scanty cytoplasm, round nuclei, and coarse chromatin with plenty of smudge cells (a). MFC show low forward scatter, dim CD20 and dim lambda, moderate CD45, CD19, CD5, CD23, CD200, and partial CD38 (40%) (b-h) and negative for CD4 and CD8.



[Table/Fig-6]: Multiple myeloma. BM shows plasmacytosis (a). MFC show CD45 negative events which are positive for cytoplasmic kappa, CD38, CD138, CD56, CD117, and HLADR, and negative for CD19 and CD20 (b-g).

expression in ATLL or subset restriction. ATLL (n=1) diagnosed on MFC presented with CD7-, CD8-, CD16-, CD56-, and CD34- but had expression of CD3+, CD4+, and CD5+. Whereas, HSTL-γ,δ cases (n=2) presented with surface CD3+, CD7+, CD56+, CD4-, CD5-, and CD8-. However, only 1 case of HSTL-γ,δ presented with CD16+. Moreover, all 3 cases were CD34- [Table/Fig-7].

Finally, MFCI was contributory in diagnosing 74 CLPD cases which on further subtyping consisted of B-cell CLPD (n=70), and T-cell CLPD (n=3). B-cell CLPD included MM (n=27), CLL (n=25), DLBCL (n=7), FL (n=3), BL (n=2), MCL (N=2), PLL (n=2), and SMZL (n=2). Moreover, one case of B-NHL could not be subtyped on MFC. Whereas, the T-cell CLPD included HSTL-γ,δ (n=2) and ATLL (n=1).

Of 70 B-cell CLPD cases, majority had MM (n=27) followed by CLL (n=25) [Table/Fig-8].

DISCUSSION

In this study, of 85 cases, 74 were diagnosed as CLPD on clinico-morphological examination, while 11 were strongly suspected cases of CLPD. On MFC, of 74 cases, 73 were confirmed and 1 was found to be false positive. While, of 11 discordant cases, majority were reactive lymphocytosis (n=9), while remaining were SLL and T-ALL (n=1 each). Other studies have reported nearly similar distribution [Table/Fig-9] [8-10].

Diagnosis		CD3+	CD4+	CD7+	CD8+	CD16+	CD34+	CD56+
ATLL (N=1)	N	1	1	0	0	0	0	0
	%	100	100	0	0	0	0	0
BL (N=2)	N	0	0	0	0	0	0	0
	%	0	0	0	0	0	0	0
CLL (N=25)	N	0	0	0	0	0	0	0
	%	0	0	0	0	0	0	0
DLBCL (N=7)	N	0	0	0	0	0	0	0
	%	0	0	0	0	0	0	0
FL (N=3)	N	0	0	0	0	0	0	0
	%	0	0	0	0	0	0	0
HSTL- γ,δ (N=2)	N	2	0	2	0	1	0	2
	%	100	0	100	0	50	0	100
MCL (N=2)	N	0	0	0	0	0	0	0
	%	0	0	0	0	0	0	0
MM (N=27)	N	0	0	0	0	0	0	22
	%	0	0	0	0	0	0	81.5
PLL (N=2)	N	0	0	0	0	0	0	0
	%	0	0	0	0	0	0	0
SMZL (N=2)	N	0	0	0	0	0	0	0
	%	0	0	0	0	0	0	0
B-NHL (N=1)	N	0	0	0	0	0	0	0
	%	0	0	0	0	0	0	0

[Table/Fig-7]: Expression of T-tube CD markers in cases diagnosed by MFC immunophenotyping.
ATLL: Adult T-cell lymphoma; BL: Burkitt's lymphoma; CLL: Chronic lymphocytic leukaemia; DLBCL: Diffuse large B-cell lymphoma; FL: Follicular lymphoma; HSTL gamma, delta: Hepatosplenic gamma delta T cell lymphoma; MCL: Mantle cell lymphoma; MM: Multiple myeloma; PLL: Pro-lymphocytic leukaemia; SMZL: Splenic marginal zonal lymphoma

CLPD cases	N	%
B-cell (N=70)		
Multiple myeloma	27	38.6
Chronic lymphocytic leukaemia	25	35.7
Diffuse large B-cell lymphoma	7	10
Follicular lymphoma	3	4.3
Burkitt's lymphoma	2	2.8
Mantle cell lymphoma	2	2.8
Pro-lymphocytic leukaemia	2	2.8
Splenic marginal zonal lymphoma	2	2.8
T-cell (N=3)		
Hepatosplenic gamma delta T cell lymphoma	2	66.66
Adult T-cell lymphoma	1	33.33

[Table/Fig-8]: Subtyping of the B- and T-cell CLPD cases as per MFC immunophenotyping.

Studies	Number of cases	Diagnosis given on MFC				
		CLPD	Reactive	HL	Non-HLM	Non-classifiable
Present study	85	74	9	-	1	1
Paul T et al., [8]	65	61	-	-	-	4
Dey BP et al., [10]	35	28	-	1	6	-
El-Sayed AM et al., [9]	50	35	9	2	4	-

[Table/Fig-9]: Comparison of distribution of diagnosis on MFC Immunophenotyping in the present study with other studies [8-10].
HL: Hodgkin lymphoma; Non-HLM: Non haematolymphoid malignancy

In this study, CD45 gating strategy was used for MFC analysis. The analysis of CD45 expression against SSC graph was helpful in isolating, distinguishing, and studying major cell lineages as majority of mature (peripheral) CLPDs had bright CD45+ with low SSC and identification of prominent and cohesive population of light chain-restricted B-Cells demonstrating monoclonality by MFC helped not only in confirming the diagnosis of CLPD but also in differentiating B-cell and T-cell CLPD. It also helped to sub-classify the B-cell NHL cases into mature and immature or precursor cases. MFCI helped in simultaneous measurement of several surface and intracytoplasmic markers on a single cell along with co-expression of various antigens to confirm and distinguish B-cell and T-cell CLPD, thus, assisting in sub-classification of CLPD.

In this study, B-cell (94.59%) CLPD mainly included MM (38.6%), CLL (35.7%), DLBCL (10%), and FL (4.3%). Whereas, the T-cell (4%) CLPD included HSTL-γ,δ (66.7%) and ATLL (33.3%). Compared to the developed nations, the key differences in the presentation of Indian CLPDs, include median age of 54 years (almost a decade less), higher male-to-female ratio, higher proportion of patients with B-symptoms (40-60%), higher frequency of DLBCL (60-70%), lower frequency of FL (<20%), and T-cell type in 10-20% [11]. The findings of this study are similar to these observations.

The MFC simultaneously measures several surface and intra-cytoplasmic markers on a single cell, thus, allowing accurate phenotypic characterisation of the analysed population. Analysis of single marker does not permit a definite cell lineage assessment but analysis with panels of antibodies allows separation of the tumours into very precise sub-types with different prognosis and treatment requirements [12]. Majority of CLPDs including CLL and FL have bright CD45 and low SSC and decreased forward scatter (FSC), whereas plasma cells are negative for CD45 with low or intermediate SSC [13,14]. The SSC corresponds to the granularity of the cytoplasm and the cells with agranular cytoplasm (lymphocytes) have low SSC. The FSC corresponds to cell size with large cells having higher FSC than smaller cells [15].

As mentioned above, this study demonstrates that MFCI analysis of CD45 expression against SSC provides a rapid and reproducible method for distinguishing major cell types. Of 74 CLPD cases, 52 presented with CD45 positivity and predominantly low SSC. In 13 cases increased FSC was observed (DLBCL, PLL, BL, and ATLL), whereas decreased FSC was seen in 39 cases (mostly CLL, MCL, FL, and HSTL-γ,δ). Similarly, Sales MM et al., observed that CLL, PLL, MCL, FL, HCL, SMZL, BL, Mucosa-associated Lymphoid Tissue (MALT) were positive for CD45 marker [16]. Other studies have reported similar findings [8-10].

Majority of mature B-cell neoplasm show a single clone of cells, expressing only one class of immunoglobulin light chain (ILC) and bright CD45. Immature B-cells lack surface ILC and are usually dim CD45 positive and sometimes CD45 negative, whereas, the reactive population of cells are polyclonal with both κ and λ expression in ratio of 1.2-2.7:1 [17]. Identification of a large relatively pure population of light chain-restricted B-Cells is fairly straightforward using FCM immunophenotyping, and is usually reflected in an abnormal κ-λ ratio [2].

In this study, identification of abnormal mature B-lymphoid cells was performed by assessment of surface ILC class restriction. Among 71 mature B-NHL cases, all presented with light chain restriction (κ restriction=49 and λ restriction=22). El-Sayed AM et al., reported light chain restriction in all the 32 cases of mature B-NHL, with 20 cases showing κ restriction and 10 cases showing λ restriction and 2 cases absence of both κ and λ [9]. Dey BP et al., reported light chain restriction in 30 out of 40 B-NHL cases. There were 15 cases of κ and λ light chain restriction each. Among the 10 cases which lacked the light chain restriction, there were 6 cases of lymphoblastic lymphomas including precursor B-NHL (n=1) and precursor T-NHL (n=5) [10].

In this study, use of MFC resulted in diagnosis and sub-classification of all the cases of CLPDs. This study demonstrated that all 25 CLL cases were monoclonal with predominantly κ chain restriction with CD19+, CD20+, CD22+, CD5+, and CD200+. Among these 25 cases, 23 presented were CD23+. It was observed that CD200 was more specific than CD23 in diagnosing CLL. Moreover, 18 cases were CD43+, and 16 were CD38+. Other studies by Paul T et al., and Stacchini A et al., have reported similar findings [8,18].

Evaluation of CD4 and CD8 expression is helpful in differentiating mature T-lymphoid neoplasm from precursor T-lymphoid neoplasm. CD4/CD8+/- with surface CD3-, cytoplasmic CD3+, and CD7+ expression is indicative of immature T-NHL, whereas CD7- and CD4+ is more typical for mature T-lymphoid neoplasm [19]. Thus, use of MFCI led to identification of mature T-lymphoid neoplasm cases by loss or aberrant (dim or variable) expression of the pan-T-cell antigen, including CD3, CD5, and/or CD7, and CD4/CD8+ expression or subset restriction. Meda BA et al., used FCMI in a precursor T-NHL case revealing pan-T-cell antigen expression with loss of the CD4 marker. Of 2 mature T-NHL cases, one was CD4+, CD5+, and CD8-, while other was CD2+, CD4-, and CD8- [20]. Similar findings are reported by another study [8].

Limitation(s)

First, the authors did not follow-up the patients for staging or review of treatment response. Second, present setting lacks cytogenetic and molecular diagnostics facilities for further diagnosis and sub-classification of CLPD as per World Health Organisation (WHO) classification which are important especially in cases of aggressive lymphomas (such as T-cell lymphomas and Burkitt's lymphoma) and determining prognosis in indolent lymphoma in CLL/SLL. Third, the authors used limited panel of antibodies, especially in T-cell lymphoma panel, for sub-classification of T-cell lymphomas.

CONCLUSION(S)

As an ancillary technique to clinico-morphologic examination, MFCI is vital for diagnosis of cases with CLPDs. MFCI provides rapid, quantitative, and multiparametric analysis of various antibodies, thus resulting in determination of sub-class and prognosis of CLPD. Moreover, it leads to rapid diagnosis of reactive hyperplasia and non haematolymphoid malignancy which may mimic CLPD on morphology and hence, difficult to diagnose based on morphology alone. Moreover, MFCI permits the diagnoses of malignancy, similar to the cases of persistent lymphocytosis in this study.

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PARTICULARS OF CONTRIBUTORS:

1. Associate Professor, Department of Pathology, Government Medical College and Hospital, Nagpur, Maharashtra, India.
2. Associate Professor, Department of Pathology, Government Medical College and Hospital, Nagpur, Maharashtra, India.
3. Assistant Professor, Department of Pathology, Government Medical College and Hospital, Nagpur, Maharashtra, India.
4. Assistant Professor, Department of Pathology, Government Medical College and Hospital, Nagpur, Maharashtra, India.
5. Associate Professor, Department of Pathology, Government Medical College and Hospital, Nagpur, Maharashtra, India.
6. Professor, Department of Pathology, Government Medical College and Hospital, Nagpur, Maharashtra, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Mukesh Waghmare,
Assistant Professor, Department of Pathology, Government Medical College and Hospital, Hanuman Nagar, Ajni Road, Medical Chowk, Ajni,
Nagpur-440003, Maharashtra, India.
E-mail: drmhwagh@rediffmail.com

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