(International Print/Online Journal)

SJIF IMPACT FACTOR: 5.565
PUBMED-National Library of Medicine ID-101739732

ISSN (Print): 2209-2870
ISSN (Online): 2209-2862

International Journal of Medical Science and Current Research (IJMSCR)
Available online at: www.ijmscr.com
Volume 4, Issue 3, Page No: 83-95
May-June 2021

Immunophenotypic Profile of Non-Hodgkin Lymphoma by Flow Cytometry and Immunohistochemistry in Peripheral Blood and Bone Marrow

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Type of Publication: Original Research Paper
Conflicts of Interest: Nil

ABSTRACT

Background: The current WHO classification has emphasized that Non-Hodgkin lymphomas (NHL) cannot be classified on the basis of morphology alone and should be supplemented by immunophenotypic studies, which can be done either by flow cytometry (FCM) or Immunohistochemistry (IHC).

Material and methods: The present study was done to compare the role of flow cytometry and immunohistochemistry and their concordance in the diagnosis and classification of various peripheral NHL. Thirty newly diagnosed cases of NHL were evaluated by FCM on peripheral blood or bone marrow aspirate samples and by IHC on bone marrow biopsy sections using a panel of monoclonal antibodies including CD19, CD20, CD5, CD10,CD23, CD79,CD45,kappa,lambda.

Results: All the thirty cases in our study comprised of B-cell NHL. These cases were classified into CLL and Non-CLL group based on morphology and primary panel. An extended panel was used for further sub-typing of non-CLL cases which comprised of FMC7, CD200, CD25, CD103, CD123, CyclinD1, BCL2, BCL6. The overall concordance rate between FCM and IHC regarding the diagnosis of various B-cell lymphomas was 86.6%. The concordance rate between FCM and IHC for all the comparable markers ranged from 76% to 100%.

Conclusion: We propose a two-tier approach for immunophenotypic analysis of newly diagnosed NHL cases by either method with a minimum primary panel including CD19, CD20, CD5, CD23, CD79, CD10, CD45 for differentiation into CLL/non-CLL group and Kappa and lambda for clonality assessment. An extended panel may be applied wherever required for further sub-typing and stratification.

Keywords: Bone Marrow, Flow Cytometry, Immunohistochemistry.

INTRODUCTION

Non-Hodgkin lymphomas (NHL) are a heterogenous group of chronic lymphoproliferative disorders originating in B, T or Natural Killer (NK) lymphocytes characterized by abnormal proliferation of monoclonal lymphocytes in peripheral blood, bone marrow, lymph node or any other lymphoid tissue and represents one of the major health problems all over the world. B-cell lymphomas represent 80% to 85% of the cases, with 15% to 20% being T-cell lymphomas; NK lymphomas are rare [1]. Morphological diagnosis of NHL is usually done on lymph node biopsy. In patients of NHL, the examination of bone marrow biopsy (BMB) is an important part of the staging procedure. However, the morphological evaluation of BMB to assess the involvement in lymphoid malignancies can be problematic [2]. Here comes the role of immunophenotypic studies into diagnostic pathology which can be done by Flow Cytometry (FCM) or Immunohistochemistry (IHC) and has allowed a more precise diagnosis and characterization of these disorders. A combination of morphologic and

immunologic findings is now the basis for the proper classification of the lymphoproliferative disorders [3]. Each of the two methods comes with its advantages and disadvantages. FCM provides a rapid diagnosis, analyzes broader array of antigens, allows quick multiparametric analysis of large number of cells and detection of expression of combination of 2 or 3 antigens on the same cell at the same time. IHC on other hand provides a visual impression of patterns of infiltration and architectural relationships between different cells. Another privilege of IHC is the long-term preservation after processing and ability to re-examine it at any time [4].

MATERIAL AND METHODS

Thirty newly diagnosed cases of NHL either by bone marrow aspirate/biopsy/lymph node biopsy/FNAC were included in the study after taking ethical clearance from institutional ethical committee. This study was conducted over a period of 2 years period from 2017 to 2019. All previously treated cases, Precursor Lymphoblastic Lymphoma/Leukemia, cases with inadequate length of bone marrow biopsies (<1.5cm), and cases that did not have infiltration on bone marrow biopsy were excluded from the study.

Bone marrow biopsy (BMB) specimens were processed by routine histological technique for paraffin embedding and morphological assessment was done on routine hematoxylin and eosin stains. Sub-typing of lymphomas was done by immunophenotyping (IPT) using a panel of monoclonal antibodies on FCM and IHC (Table 1). Peripheral blood/bone marrow aspirate was processed on FCM. FCM was performed on 8 Color Flow cytometer BD FACS Canto II (Becton Dickinson, San Jose, CA) [5]. List mode data was acquired and analyzed by FACS Diva software. Expressions of any gated events were plotted on the side scatter (SCC)/CD19 plots and SCC/CD3 plots. IHC was performed on representative sections of BMB for a panel of immunohistochemical markers [6]. Immunophenotypic diagnostic criteria depending upon the expression of antibodies was used for subtyping of chronic lymphoproliferative disorders (Table 2)

Statistics: SPSS 20.0 statistical software was used for statistical analysis. Kappa statistics were applied to find the agreement between comparable markers. Other appropriate statistical tests were also done. Difference between groups were considered significant only when p-value was <0.05.

RESULTS

The patient's age ranged from 21-86 years with a mean age of 56.43 years. On complete blood examination majority of the cases (66.6%) had elevated absolute lymphocyte count and atypical lymphocytosis on peripheral blood film. Most common clinical presentation (63.3% cases)was lymphadenopathy along with hepatomegaly or splenomegaly. On bone marrow aspiration, majority of the cases (63.3%) were reported as chronic lymphoproliferative disorder. All the cases were further subjected to bone marrow biopsy and immunophenotyping by IHC and FCM. Percentage of gated lymphocytes was calculated on FCM which ranged from 10-90%. Varied patterns of bone marrow infiltration were observed on bone marrow biopsy including diffuse (most common), nodular, interstitial and mixed. Cases were initially classified as CLL or non-CLL (NHL) group on the basis of morphology which were further typed with the help of immunophenotypic expression on FCM and IHC. Expression of CD5, CD23, dim CD79 and weak surface immunoglobulin positivity was seen as the most significant findings in the CLL group. In the non-CLL group expression of CD5, positive or negative CD23, strong CD79 and strong surface immunoglobulin expression was observed as the most common finding.

Classification of cases: On FCM 10/30 cases were CLL and 20/30 cases were reported as non-CLL. While on IHC 12/30 cases were CLL, a slightly higher number than that reported on FCM and 18/30 cases were non -CLL (Table 3 & Figure 1).An extended panel was applied for sub-typing of non-CLL cases comprising of FMC7, CD200, CD25,CD103, and CD123 on FCM and CyclinD1, BCL2 and BCL6 on IHC. Non –CLL group of 20 cases on FCM comprised 3 cases of mantle cell lymphoma (MCL), and 1 case each of as marginal zone lymphoma (MZL) and hairy cell leukemia (HCL).15/20 cases were not further sub-typed and were reported as B-NHL. Non-CLL group (n=18) on IHC comprised 4 cases of MCL, 3 cases of follicular lymphoma (FL) and 2 cases of diffuse large B cell lymphoma (DLBCL). 9/18 cases were not further

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sub-typed and were reported as B-NHL. Total concordance between FCM and IHC regarding CLL and Non-CLL was 86.6% with a P-value of 0.000 (<0.001), making this correlation highly statistically significant. (Table 4 & Figure 2)

Agreement between FCM and IHC was seen in CLL, NHL (unclassified) and Mantle cell lymphoma. Agreement was highest regarding CLL; 83.3% followed by Mantle cell lymphoma;75% and NHL (unclassified); 60%.No agreement could be assessed for atypical CLL, MZL, HCL, FL, DLBCL, since these cases were diagnosed exclusively either on FCM or IHC. (Table 5& Figure 3)

CORRELATION BETWEEN FCM AND IHC IMMUNOPHENOTYPING:

Taking IHC as the gold standard test, appropriate statistics were applied including KAPPA statistics and concordance rate for every marker was calculated between FCM and IHC. Regarding CD45, CD19, CD20, CD30, CD3, CD7, CD4, CD8 and CD56, no statistics were computed because their expression was constant on FCM and IHC. (Table 6). Correlation was found for the comparable markers including CD5, CD23, CD79, CD10, KAPPA and LAMBDA. Agreement between various comparable markers ranged from 76-100%.CD5 expression had a fair agreement having a concordance rate of 76.6% and a P-value of 0.003, making this correlation statistically significant. For CD23 very good agreement was found with a concordance rate of 86.6%, this correlation was statistically significant with a P-value of <0.001. Regarding CD10 absolute agreement was seen with a concordance rate of 100% and a P-value of <0.001 making this correlation highly statistically significant. For CD79 correlation was statistically non-significant with a P-value of 0.414. (Table 7)

Regarding light chain restriction a considerable discordance was noted in detecting kappa and lambda chain expression. Many of the cases were non-specific for kappa (13/30) as well as lambda (15/30) on IHC, whereas clonality assessment was much easier on FCM. Total agreement regarding lambda could not be compared for all the cases. Fifteen cases on IHC showed non-specific staining hence, excluded and not compared. Concordance for comparable cases (15 cases) was found to be 80%. This correlation was statistically non-significant (P-value >0.05). Similarly agreement regarding KAPPA could not be compared for all the cases. Thirteen cases on IHC showed non-specific staining (13 cases), hence excluded and not compared. Concordance for comparable cases (17 cases) was found to be 100%. This correlation was statistically significant (P-value <0.05).

DISCUSSION

Modern pathology relies on IPT for accurate classification of lymphomas. The current WHO classification suggests classification of lymphomas on the basis of morphology along with supplementation with IPT. In the current study, we compared the role of FCM and IHC in the diagnosis and classification of NHL and their concordance rate.

We observed expression of CD5, CD23, dim CD79 and weak surface immunoglobulin positivity as the most significant findings in the CLL group. In the non-CLL group expression of CD5, positive or negative CD23, strong CD79 and strong surface immunoglobulin expression was observed as the most common finding. In our study, a screening panel comprising of CD5, CD23, CD79 and SIg was sufficient to distinguish between CLL from Non-CLL group. These findings were comparable to **Dewan K et al [7]** who divided CLL and non-CLL groups on the basis of positive reactivity to CD5, CD23, weak SIg reactivity and non-reactivity of FMC7, CD79.

The overall concordance between FCM and IHC in the diagnosis of NHL in our study was 86.6% which was in concordance to studies done by **Martinez A et al (87.2%) [8]**, **Sah SP et al (88%) [2], EI-Sayed A et al (88%) [9] and Bezerra A et al (81%) [10]** However; a lower concordance rate was observed by **Naughton MJ et al** (60%) [11] and **Duggan et al (78%) [12].** An older technique and limited panel of antibodies used could account for lower concordance rates observed in these studies. A higher concordance rate 93% was obtained by **Dewan K et al [7]** and 100% by **Biesemier KW et al [13]**. This difference in concordance rates may be attributed due to sample processing, difference in the sample size, and the panel of antibodies used .

There were few discrepancies regarding the subtyping of B-lymphomas in our study. **Complete agreement** was seen in 18 out of total 30 cases. Among these 7 were NHL, 8 CLL, and 3

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MCL. Agreement between FCM and IHC was highest regarding CLL (83.3%) followed by MCL (75%) and NHL (60%). Our findings are slightly different from that observed by **EI-Sayed A et al [9]** who found 91.2% concordance between FCM and IHC in case of B-NHL and 100% in CLL and MCL.

Partial agreement was seen in 10 cases in which further typing of NHL was not done either on FCM or IHC. Regarding CLL, IHC was able to diagnose a greater number of cases than FCM. This discordance was due to negative expression of CD5 in three cases of CLL on FCM, while these cases had positive CD5 expression on IHC, hence diagnosed. One case of NHL on FCM was reported as MCL on IHC due to its dim CD23 expression on FCM, while CD23 was negative on IHC, and CyclinD1 was positive, hence confirming the diagnosis. Two cases of NHL on FCM were sub-typed as FL on IHC due to morphological assessment and positive expression of BCL2. Similarly, two cases of NHL on FCM were reported as DLBCL on IHC due to morphological assessment and positive BCL6 expression. The similar findings were observed by **Bezerra A et al [10]**, who found IHC a better tool to assess CD5 expression in diagnosing CLL/SLL and CyclinD1 for confirmation of MCL. Regarding FL and DLBCL, they observed that assessment of CD10 expression and availability of BCL2 and BCL6 were factors favoring IHC as a better modality in diagnosing these types of lymphomas. Regarding two cases of NHL which were unclassified on IHC, one was sub-typed as HCL on FCM due to availability of HCL markers: CD23, CD103, CD123 exclusively on FCM in our institute. Another case of NHL was diagnosed as MZL due to difference in expression of CD5, it was CD5 positive on IHC while negative on FCM.

No agreement was seen between FCM and IHC in two cases. These two cases on FCM were diagnosed as atypical CLL while they were reported as CLL and FL respectively on IHC. This discordance was due to negative CD5 expression on FCM and positive CD5 expression on IHC. One of the cases also showed positive CD10 and BCL2 expression on IHC and hence was reported as follicular lymphoma.

Hence in our study IHC was found better to diagnose CLL, MCL, FL and DLBCL while FCM was a better modality to diagnose HCL, MZL and atypical CLL. The similar findings were observed by **Dewan K et al [7]**. In their study, Follicular lymphoma was diagnosed on IHC while it was missed on FCM. **Sah S P et al [2], Martinez A et al [8]** and **Dunphy CH et al [14]** in their studies had different observations. In their study FCM was a better technique to diagnose CLL, MCL and FL. **El-Sayed A et al [9]** observed that FCM was able to diagnose DLBCL while it was not diagnosed on IHC.

The discordance between the diagnosis in our study was due to negative CD5 expression on FCMand could be attributed to the presence of heterogenous populations of lymphocytes seen in partial involvement of the marrow by lymphoma cells or presence of numerous residual non-neoplastic lymphocytes among the neoplastic cells as reported in literature.

Taking IHC as the gold standard test, appropriate statistics were applied including KAPPA statistics and concordance rate for every marker was calculated between FCM and IHC.

Correlation was found for the comparable markers including CD5, CD23, CD79, CD10, KAPPA and LAMBDA. Agreement between various comparable markers ranged from 76-100%, similar to studies by **Dewan K et al [7] and Biesemier KW et al** [13[who observed concordance between 80-100% and 76-100% respectively for various immunological markers. CD5 expression had a fair agreement having a concordance rate of 76.6% and a P-value of 0.003, making this correlation statistically significant. A lower concordance rate of 80% as regards CD5 was also observed by **Biesemier KW et al [13].** For CD23 a significant agreement was found with a concordance rate of 86.6%, p-value <0.001, which was slightly lower than that observed by **Dewan K et al (90%) [7]**. Regarding CD10 absolute agreement was seen with a concordance rate of 100% and a P-value of <0.001 making this correlation highly statistically significant. For CD79 correlation was statistically non-significant with a P-value of 0.414.

Regarding light chain restriction a considerable discordance was noted in detecting kappa and lambda chain expression. It was difficult to interpret the immunoglobulin (Ig) light chain detection by IHC because of background staining in paraffin embedded blocks and antigenic destruction by fixation and decalcification of BMB specimens. Many of the cases were non-specific for kappa (13/30) as well as

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lambda (15/30) on IHC, whereas clonality assessment was much easier on FCM. The similar findings were observed by **Leers MP et al** [15] who pointed out lack of contrast between surface Ig staining and extracellular Ig staining as a major drawback in IHC detection of monoclonality, and also by **Abdel-Ghafar A et al [16]** who observed destruction of some of the antigenic epitopes by fixation and decalcification process. A similar observation was also made by **Dunphy CH et al [14]** who highlighted the difficulty arising due to weak expression of antigen in paraffin tissue because of variations in tumor preservation and fixation. Since the staining was non-specific on IHC, so these cases could not be compared.

Based on our results, we propose a basic approach in immunophenotypic analysis of all newly diagnosed cases of NHL. FCM on peripheral blood or bone marrow and IHC on bone marrow biopsy specimens can be used for IPT analysis. The screening panel should comprise following markers CD19,CD20 (B-cell), CD3, CD7, CD4,CD8 (T-cell), CD45 (common leucocyte antigen), CD30 (Hodgkin lymphoma), CD56(NK cell), CD23,CD79,CD5,CD10 (for further categorization) KAPPA and LAMBDA (monoclonality assessment). A limited primary panel comprising CD5, CD23, CD79 and sIg can be used to differentiate between CLL and non-CLL. Using the screening panel, diagnosis of CLL, MCL, MZL could be made in our study. Diagnosis of other types of NHL; atypical CLL, HCL, FL, DLBCL and confirmation of MCL in few cases requires an extended panel comprising of CD200,FMC7(atypical CLL) CD25,CD103,CD123 (HCL), cyclinD1 (MCL), BCL2 (FL) and BCL6 (DLBCL). T-cell and NK-cell NHL should be further evaluated if positive for any T-cell or NK-cell marker, which was not seen in our study in any of the case.

CONCLUSION

Based on our results we conclude that: Role of immunophenotyping by either FCM or IHC in diagnosis of CLPD is indispensable. Both the techniques are equally effective in diagnosing CLL. In our study we observed a significant concordance between FCM and IHC in categorization of cases into CLL and NHL with a limited panel of antibodies. IHC was a better tool for sub-typing of NHL cases, while flow cytometry was found to be more sensitive in assessment of monoclonality.

Hence, we propose a two-tier approach for immunophenotypic analysis of newly diagnosed CLPD case by either method with a minimum primary panel including CD19, CD20, CD5, CD23, CD79, CD10, CD45, KAPPA and LAMBDA for clonality assessment and differentiation into CLL/non-CLL group. An extended panel may be applied wherever required for further sub-typing and stratification.

TABELS

FCM		**IHC**	
Primary panel	**Secondary panel (exclusive on FCM)**	**Primary panel**	**Secondary panel (exclusive on IHC)**
CD3	FMC7	CD3	Cyclin D1
CD5	CD200	CD5	BCL2
CD7	CD25	CD7	BCL6
CD4	CD123	CD4	
CD8	CD103	CD8	
CD19	CD38	CD19	
CD20		CD20	
CD23		CD23	
CD79		CD79	

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CD10		CD10	
CD30		CD30	
CD45		CD45	
CD56		CD56	
KAPPA		KAPPA	
LAMBDA		LAMBDA	

Table 1- A diagnostic panel of antibodies (primary and secondary panel) was used for IHC and FCM analysis.

Table-2 Immunophenotypic diagnostic criteria depending upon the expression of antibodies was used for subtyping of chronic lymphoproliferative disorders.

	CD 19	**CD 20**	**CD 23**	**CD 5**	**CD 79**	**CD 10**	**CD 25**	**CD 103**	**FMC 7**	**CD 200**	**BCL 2**	**BCL 6**	**CYCLIND1**
CLL	+	+	+	+	Dim +	-	-	-	Dim +	++	-	-	-
ATYPICAL CLL	+	+	+	-	Dim +	-	-	-	Dim +	+	-	-	-
NHL (unclassified)	+	+	+	+	+	-	-	-	+	-	-	-	-
MCL	+	+	-	+	+	-	-	-	+	-	-	-	+
MZL	+	+	-	-	+	-	-	-	+/-	-	-	-	-
HCL	+	+	-	-	+	-	+	+	+	+	-	-	-
FL	+	+	-	-	+	+	-	-	-	-	+	-	-
DLBCL	+	+	-	+	+	+	-	-	-	-	-	+	-

Table3: correlation of CLL and non-CLL groups between FCM and IHC

		IHC		**Total**	**P-value**
		NON-CLL	CLL		
FCM	NON-CLL	17	3	20	
	CLL	1	9	10	
Total		18	12	30	**0.000**

Total concordance between FCM and IHC regarding CLL and NON-CLL was 86.6% with a P-value of 0.000 (<0.001) , making this correlation highly statistically significant.

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Figure 1: Showing Correlation of CLL and Non-CLL between FCM and IHC

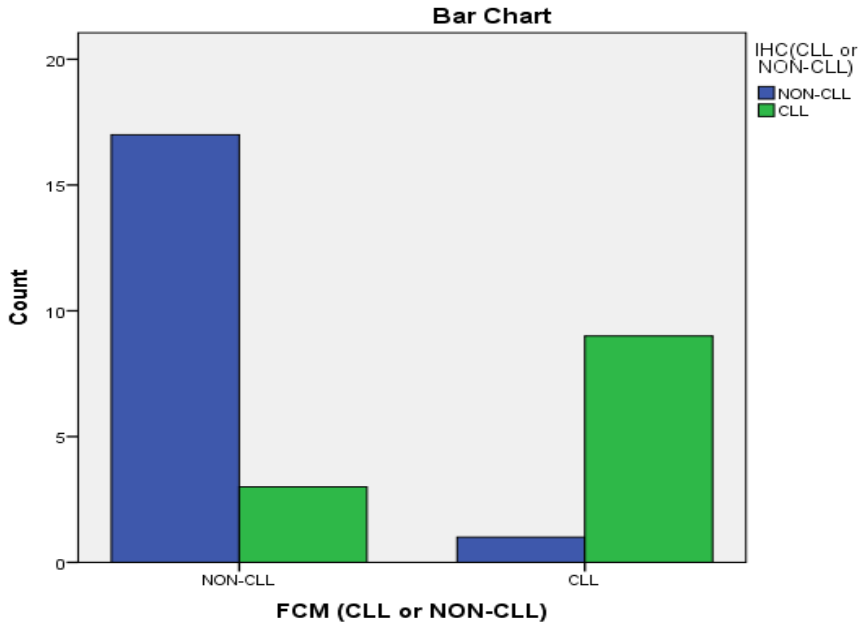

Table 4- Correlation was done between B-cell lymphoma subtypes diagnosed by FCM and IHC as shown in the table:

B-CELL LYMPHOMA	NUMBER OF CASES ON FCM (%)	NUMBER OF CASES ON IHC (%)
CLL		
CLL/SLL	08 (26.6%)	12 (40%)
Atypical CLL	02 (6.66%)	00 (0%)
NON-CLL		
B-NHL (unclassified)	15 (50%)	09 (30%)
Mantle cell lymphoma	03 (10%)	04 (13.33%)
Follicular lymphoma	00 (0%)	03 (10%)
Diffuse large B cell lymphoma	00 (0%)	02 (6.66%)
Marginal Zone lymphoma	01 (3.33%)	00 (0%)
Hairy cell leukemia	01 (3.33%)	00 (0%)
TOTAL CASES	**30 (100%)**	**30 (100%)**

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Figure 2: Graph Showing correlation between FCM and IHC

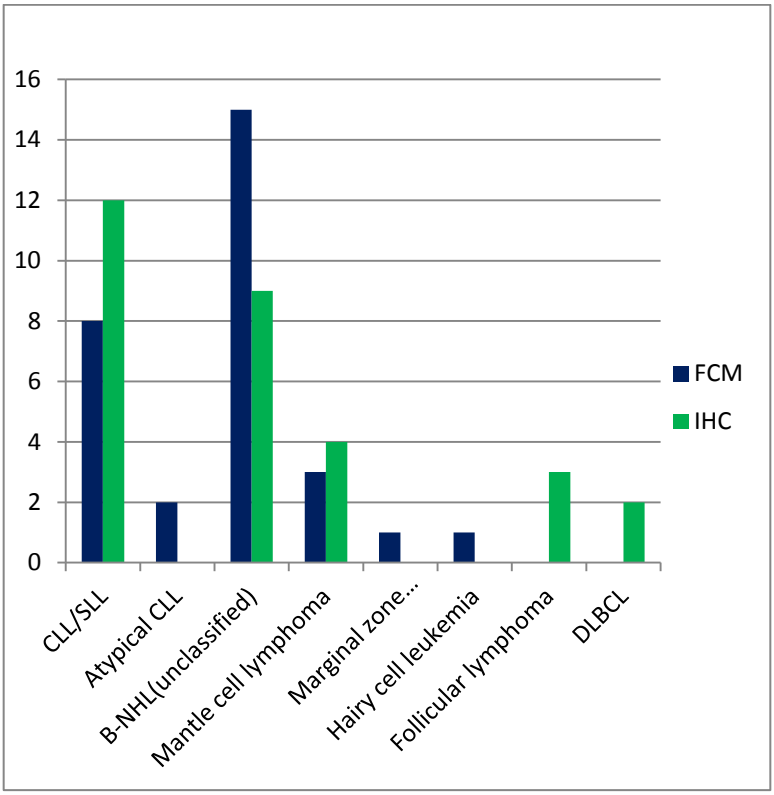

Table5: Agreement between FCM and IHC regarding diagnosis

B CELL LYMPHOMA	**% AGREEMENT BETWEEN FCM AND IHC**
CLL/SLL	83.3%
Mantle cell lymphoma	75%
B-NHL(unclassified)	60%
Atypical CLL	NA
MZL	NA
HCL	NA
FL	NA
DLBCL	NA

(NA= Not assessed)

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Figure 3: Percentage of diagnosis with agreement between FCM and IHC

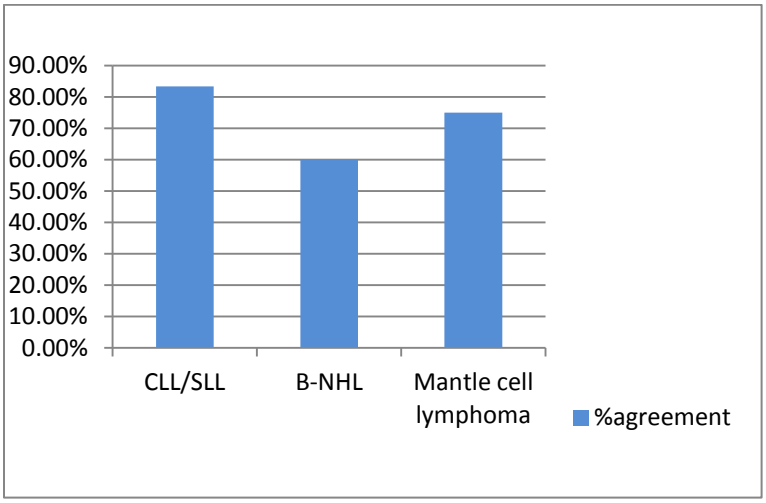

Table 6: Agreement of the markers (primary panel): Kappa statistics

Antibody	Number of positive cases on FCM (n=30)	Number of positive cases on IHC (n=30)	Positive agreement between FCM and IHC	Negative agreement between FCM and IHC
CD19	30	30	30/30	-
CD20	30	30	30/30	-
CD30	00	00	-	30/30
CD3	00	00	-	30/30
CD7	00	00	-	30/30
CD4	00	00	-	30/30
CD8	00	00	-	30/30
CD56	00	00	-	30/30
CD45	30	27	-	27/30

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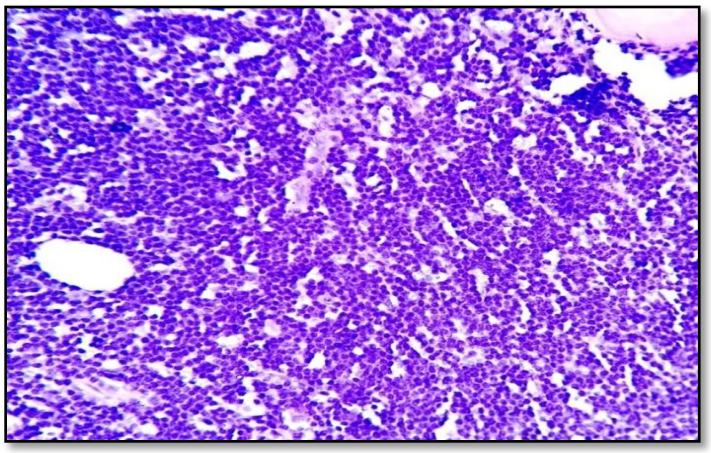
Table 7: Agreement of the markers (primary panel): Kappa statistics

Antibody (n=30)	Number of positive cases on FCM	Number of positive cases on IHC	Positive agreement between FCM and IHC	Negative agreement between FCM and IHC	P-value	Concordance between FCM and IHC (%)
CD23	20	20	18/20	8/10	**<0.001**	86.6%
CD79	25	27	23/27	1/3	0.414	80%
CD5	18	25	18/25	5/5	**0.003**	76.6%
CD10	07	07	7/7	23/23	**<0.001**	100%

CHRONIC LYMPHOCYTIC LEUKEMIA



Bone marrow biopsy showing diffuse infiltration of bone marrow by Lymphoid cells of small size with scant cytoplasm and inconspicuous nucleoli, H&E(400x)

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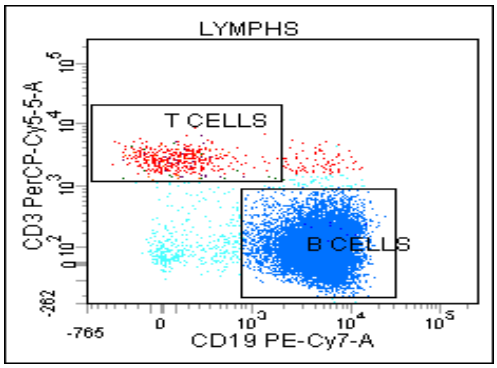
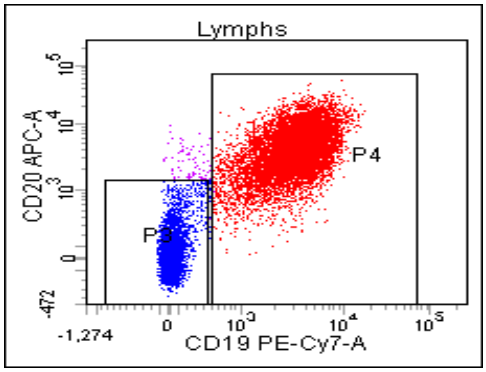
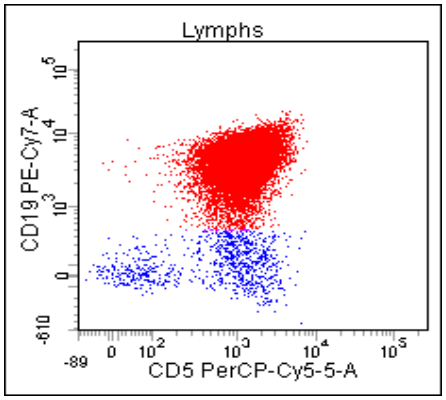
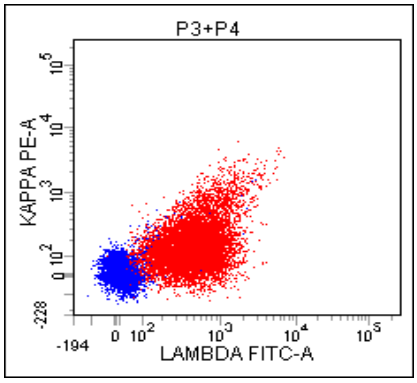
FLOW CYTOMETRY



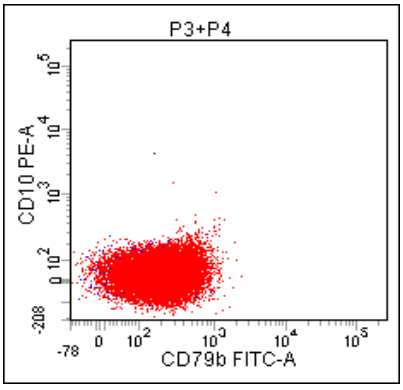


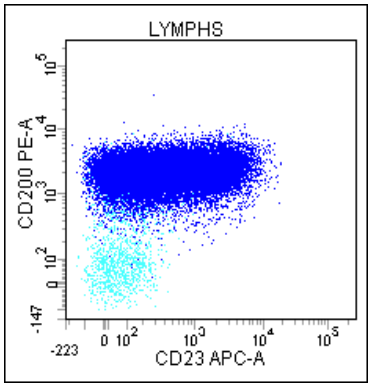
CD19+ , CD20+ B-CELL POPULATION





CD5+ , CD19+ B-CELL POPULATION WITH LAMBDA CHAIN RESTRICTION





CD10- CD79- CD23+ CD200+ B-CELL POPULATION

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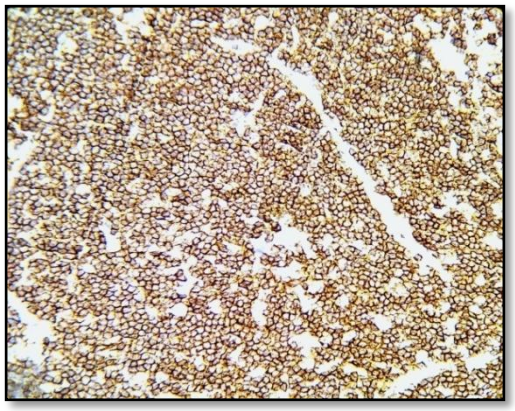
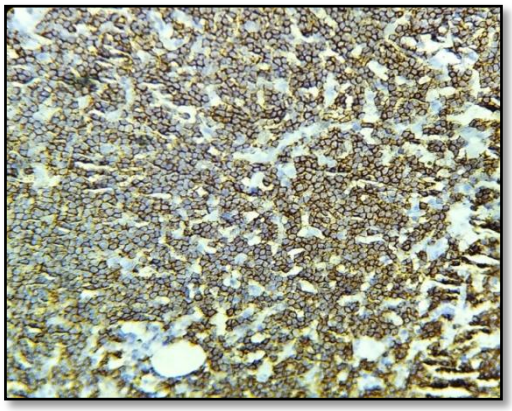
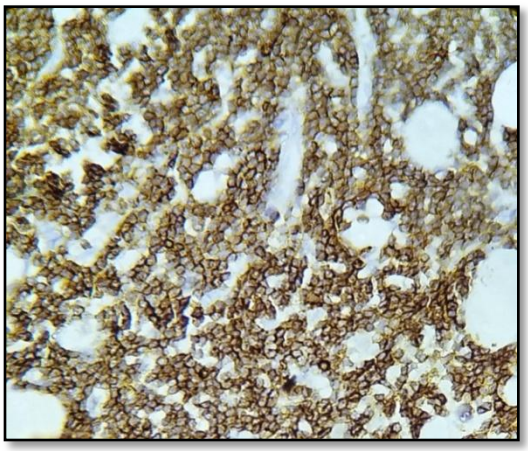
IMMUNOHISTOCHEMISTRY



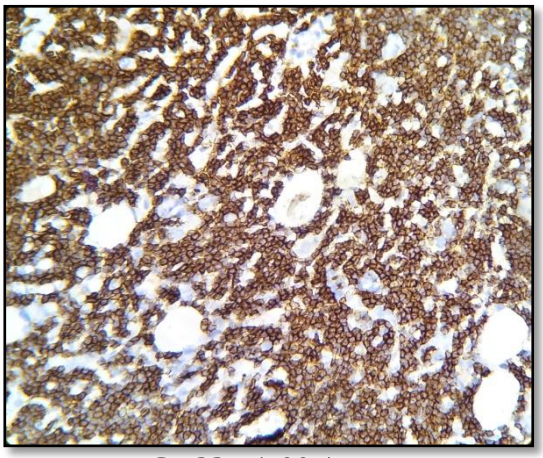
CD19+ (400x)



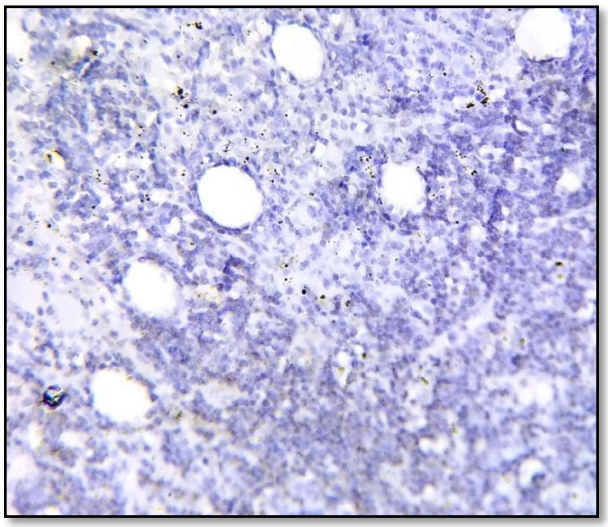
CD20+ (400x)



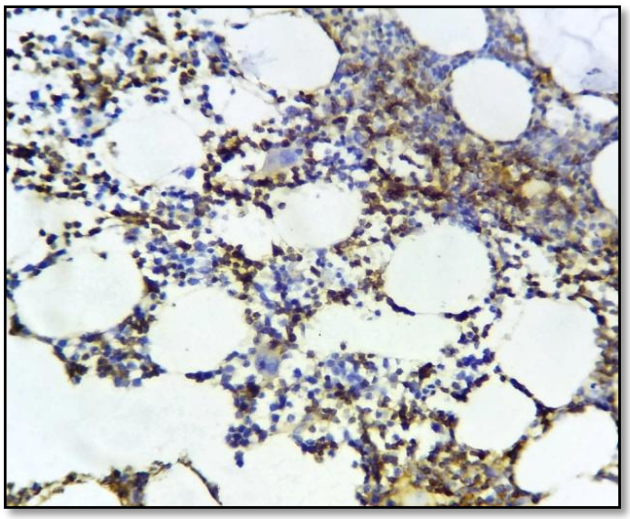
CD5+ (400x)



CD23+ (400x)



CD79- (400x)



LAMBDA, NON-SPECIFIC (400x)

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