*Journal of the Egyptian Society of Haematology & Research, Vol. 8, No. 1, March: 1-9, 2012*

# A Suggested Algorithm for Detection of Bone Marrow Involvement in Diffuse Large B-Cell Lymphoma (DLBCL)

AZZA M. KAMEL, M.D.; ENAS S. ESSA, M.D.*; HEBA S. MOUSSA, M.D.; AMR M. ZAHER, M.D.; YASSER H. ELNAHAS, M.D.** and HAGAR A. ALAGIZY, M.D.

*The Departments of Clinical Pathology, NCI, Cairo University, Clinical Pathology* and Clinical Oncology**, Faculty of Medicine, Menofiya University, Shebein El-Kom, Menofiya, Egypt*

## ABSTRACT

Diffuse large B-cell lymphoma (DLBCL) is defined by the World Health Organization (WHO) as a heterogeneous entity, encompassing morphologic and genetic variants, and variable clinical presentations and outcomes. It accounts for approximately 30% of NHL and 80% of all aggressive lymphomas. In Egypt, DLBCL is the most common subtype of NHL; it represents about 49% of NHL presenting to the National Cancer Institute, Cairo University. In DLBCL patients, bone marrow (BM) involvement portends a poor prognosis and correlates with poor survival. Thus, assessment of BM status in patients with DLBCL is critical for proper staging and planning of optimal therapeutic strategies.

Although the traditional light microscopic examination of the BM aspirate (BMA) and trephine BM biopsy (BMB) has been considered the gold standard technique for routine BM evaluation in NHL patients, additional laboratory techniques have been recruited like flowcytometric immuno-phenotyping, immunohistochemical staining and/or molecular studies to improve their assessment capabilities. In this work we tried to re-stage DLBCL patients by assessment of BM status, by morphology / immunohistochemictry as well as molecular testing, to detect possible occult BM involvement, hence verifying the value of incorporating this technique in the initial routine testing for the BM.

***Patients and Methods:*** A total of 45 cases newly diagnosed DLBCL patients were included in the study, during the time period from July 2004 to June 2005. They were referred from the Medical Oncology Department to the Clinical Pathology Department in Menofeyia University Hospital for staging, then to Clinical Pathology Department National Cancer Institute, Cairo University for BM morphology/Immunohistochemistry re-evaluation and molecular testing for Immunoglobulin heavy chain (IgH) gene rearrangements on DNA. The main inclusion criterion in this study was a negative bone marrow aspirate for evidence of infiltration by lymphoma cells; 41 cases were BMA negative. The 4 BMA positive cases were used as positive controls for both techniques.

***Results:*** Cases were 18 females and 23 males with an age range of 21-84 with a median of 55 years. BMB/IHC showed an overall detection rates of 11/41 (26.8%) versus molecular testing that showed 26/41(63.4%). Twenty four cases were negative by BMB/IHC, when molecularly tested 10/24 cases were positive (41.7%).

A total of 10/41 cases were negative by both methods. Two cases showed IgH rearrangements in the peripheral blood while the BM was negative. In respect to stage: Stage I cases, IgH rearrangements were detected in (63.2%) of cases, in Stage II in 50% and in Stage III in 75% of cases.

We present an algorithm for investigating stages I, II and III aiming at stressing the importance of ancillary testing and probably restaging of a sizable number of DLBCL cases.

In Conclusion, our results confirm that PCR based IgH gene rearrangement analysis is a sensitive and specific method for demonstrating B cell clonality in BMA and PB. It should be considered as an additional tool to properly stage DLBCL cases according to the suggested algorithm.

***Key Words:*** *DLBCL – IgH gene rearrangement.*

## INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is defined by the World Health Organization (WHO) as a heterogeneous entity, encompassing morphologic and genetic variants, and variable clinical presentations and outcomes **[1]**. It accounts for approximately 30% of NHL **[2]** and 80% of all aggressive lymphomas **[1]**. In Egypt, DLBCL is the most common subtype of NHL; it represents about 49% of NHL presenting to the National Cancer Institute, Cairo University **[3]**. In patients with DLBCL, bone marrow (BM) involvement portends a poor prognosis and correlates with poor survival **[4,5]**. Thus, assess-

ment of BM status in patients with DLBCL is critical for proper staging and planning of optimal therapeutic strategies. Several previous studies on DLBCL patients showed variable frequencies (11-27%) of BM involvement at diagnosis time **[4-8]**. This discrepancy may partially reflect differences in study populations but also may be partially attributed to variability in laboratory techniques used in these studies for BM assessment. Although the traditional light microscopic examination of the BM aspirate (BMA) and trephine BM biopsy (BMB) has been considered the gold standard technique for routine BM evaluation in NHL patients, several centers are recruiting additional laboratory techniques like flowcytometric immunophenotyping, immunohistochemical staining and/or molecular studies to improve their assessment capabilities **[9-16]**. However, in the routine practice converse results, potential pitfalls and technical limitations are still reported on using these ancillary techniques for BM assessment **[17-19]**; thus many of them are not yet appropriately validated.

In this respect, this work tried to re-stage DLBCL patients by assessment of BM status, using IgH chain rearrangement as a molecular technique, to detect possible occult BM involvement, hence verifying the value of incorporating this technique in the initial routine testing for the BM rather than using it in the morphologically ambiguous cases only. Also, an algorithm of using standard and ancillary laboratory testing for staging DLBCL patients is suggested to be introduced.

## PATIENTS AND METHODS

A total of 45 newly diagnosed DLBCL patients were included in the study, they were referred from the Medical Oncology Department to the Clinical Pathology Department in Menofyia University Hospital for staging, then to Clinical Pathology Department National Cancer Institute, Cairo University for morphologic re-evaluation and molecular testing during the time period from July 2004 to June 2005. They included 19 females and 26 males with an age range of 21-84 with a median of 55 years. Patients were diagnosed according to the standard routine clinical, radiologic and laboratory tests. Standard staging work-up included thoracic, abdominal, and pelvic computed tomography scans, as well as bone marrow biopsy and aspiration. The main inclusion criteria in this study was a negative bone marrow aspirate for evidence of infiltration by lymphoma cells; 41 cases fulfilled this criterion (BMA negative) but 4 cases showed frank bone marrow involvement by BM aspiration (BMA positive) and were used as positive control. They comprised one female, 60 years, stage I and three males, age 27, 53 and 62 years, stage III.

*BM morphology:*

BM biopsies were performed for all DLBCL BMA negative cases at first diagnosis. All trephines were fixed in buffered 10% formal-saline for 24 hours and then decalcified using formic acid-sodium citrate for 48 hrours. Samples were then embedded in paraffin, routinely processed and sections stained with Haematoxylin and Eosin (H&E). The adequate trephine length was in the range of 1.5cm to 2.0cm; shorter than 1.2 cm in section was considered inadequte **[20]**.

Standardized criteria were used to classify trephine biopsy samples as positive, negative or suspicous **[21]**.

*Immunohistochemistry:*

Immunohistochemical analysis was performed on sections from formalin-fixed decalcified paraffin-embedded (FFDPE) trephine biopsies which were immunostained using the monoclonal antibodies CD20, CD45RO, LCA, and the immunoperoxidase staining kit [DAKO Envision™+System (HRP Horse raddish peroxidase),, Peroxidase (DAB), Dako Cytomation, Inc. 6392 Via Real. Carpinteria, California 9013 USA].

The presence of B-cell aggregates was classified as abnormal or malignant when there were large numbers of aggregates, aggregates were large-sized, or contained disproportionate numbers of larger cells. Control slides were prepared for comparison.

*Molecular studies:*

Samples for molecular studies were obtained from BMA and/or PB.

DNA extraction was performed by salting out technique **[22]**. To verify the quantity and integrity of the DNA, all samples were, measured on the nano drop spectrophotometer (ND

1000, nanodrop technology, Thermo Fisher Scientific Inc.) at 260 and 280 wavelengths, run on gel and amplified with the house keeping gene ß-globin (Table 1).

*Immunoglobulin heavy chain IgH gene rearrangement analysis:*

Analysis of IgH gene rearrangements was performed by semi-nested PCR (sn-PCR), using framework regions (FR), FRII-$V_H$ and FRIII-$V_H$-specific primer amplifications (Table 1). In addition, the consensus FRIc primer directed against a highly conserved region in the FRI region shared by the 7 $V_H$ families was employed for the FRI-JH amplification using the same methodology described for the two other VH framework-specific primers.

For each initial amplification, 0.5µg of extracted DNA template, was used in a total of 25µL PCR reaction mixture that contained 0.5µmol of primers, 100µmol dNTPs, 10mmol Tris-HCl (pH 8.3), 1.5mmol $MgCl_2$, 50mmol KCl, and 2U Native Taq DNA polymerase (Finnzymes) and was subjected to 45 cycles of PCR amplifications. For all PCR amplifications, an initial step was set for 6 minutes at 94°C to denature the DNA templates and to activate the Taq enzyme. Then the PCR cycles consisted of denaturation at 94°C for 60 sec; annealing, for 60 sec, at 63°C for FR1c/LJH, at 50°C for FR2a/LJH and at 55°C for FR3a/LJH; followed by extension at 72°C for 60 sec with a 10 minutes final extension step. For reamplification of the IgH gene PCR products, one µl of the first round was used as a template and subjected to another 35-cycles PCR amplification using VLJH primer. Then, 10µL of each amplified product was separated on 3% agarose gel at 100 volt for 30 minutes and visualized by staining with ethidium bromide.

Clonal rearrangements of IgH chain genes resulted in one or two predominant amplification products within the expected range of size; FR1c/JH 330 to 350 bp, FR2a/JH 230 to 270 bp, and FR3a/JH 70 to 110 bp, *ß*-globin gene: HBG7-F, HBG7-R was at 300 bp. respectively **[23-26]**, (Fig. 1A,B,C).

All standard precautions were taken to guard against cross-contamination of amplified DNA. In each run, a negative (sterile water [blank]) control was systematically included. Positive control sample (Pre-B acute lymphobalstic leukemia) was systematically included in each run.

*Treatment protocol:*

Patients were treated with Cyclophosphamide, Doxorubicin, Vincristine and Prednisolone (CHOP) **[27]** or variations of CHOP chemotherapy protocols **[28,29]**. Response was assessed according to conventional criteria; complete response was confirmed by the disappearance of all lesions, determined by two observations, not less than 4 weeks apart. Partial response was defined as 50% or more decrease in total tumor size that has been measured to determine the effect of therapy by two observations, not less than 4 weeks apart. No response was defined as <50% decrease in total tumor size, while progressive disease was reported by ≥25% increase in the size of the tumor.

Follow-up period ranged from 16-80 months with a median of 34 months.

## RESULTS

The efficiency of BMB morphology and immunohistochemistry in detection of marrow involvement in BMA negative cases is presented in (Table 2).

*Molecular studies:*

IgH rearrangements were detected in all 4 BMA positive cases as well as 26/41 BMA negative cases (63.4%). FRI was the most commonly encountered in 21 cases, whether alone or in combination with other FRs, followed by FRIII (12 cases), then FRII (6 cases). The most frequent combination was FRI with FRIII (Table 3). In the BMA positive group, 3 cases had the FRI FRII FRIII combination while one case had the FRI FRII combination.

All BMB positive cases were positive for IgH rearrangement (8/8), as well as all BMB/IHC cases (11/11). In the BMB suspicious group, 7/11 (63.6%) were positive, in BMB negative group, 6/16, (37.5%) were positive; while in the BMB inadequate group, 5/6 (83.3%) were positive.

Molecular results in relation to disease stage are shown in (Table 4).

Stage I cases were 19/41 (46.3%), with 7 cases stage IE (extra nodal disease). IgH rear-

rangements were detected in 12/19, (63.2%) of whom 5/7 (71.4%) were extra nodal cases. Only 4/19 cases were negative by both morphologic/IHC and molecular methods.

Stage II were 10/41(24.4%) cases with 1/10 case stage IIE. IgH rearrangements were detected in 5/10 (50%). In this group, 3/10 cases were negative by both methods including the stage IIE case.

Stage III cases presented 12/41 cases, none were extranodal. IgH rearrangements were detected in 9/12 (75%) and the remaining 3/12 were negative by both methods.

A total of 10/41 cases were negative by both methods.

Comparing detection rates of BMB/IHC versus molecular testing showed an overall detection rates of 11/41 (26.8%) versus 26/41 (63.4%) respectively. Twenty four cases were negative by BMB/IHC, when molecularly tested 10/24 cases were positive (41.7 %). (In view of stage, in stage I, it was 15.7% vs. 63.7%), stage II 10% vs. 50% and in stage III 41.7% vs. 75%).

Two cases showed IgH rearrangements in the peripheral blood while the BM was negative. One of them was stage II with a suspicious BMB but IHC showed polyclonality. The other was stage III with also suspicious by morphology but showed B clonality by IHC.

Here we present an algorithm for investigating stages I, II and III aiming at stressing the importance of ancillary testing and probably restaging of a sizable number of DLBCL cases (Fig. 2).

Clinical follow-up was available for 32/45 cases. All 4 BMA positive cases relapsed over a variable period of time (7-30 months). The 28/41 BMA negative did not show a particular treatment response pattern as regards the clinical, standard lab, BMB/IHC or molecular testing results. Follow-up period ranged from 16 to 80 months. There were 18 relapses (R), 5 partial response (PR), and 3 in complete remission (CR). Their distribution among the stages was as follows: Stage I: 10 R, 1 PR and 1CR (12/19cases), stage II: 5 R, 3 PR, 1CR (9/10 cases) and stage III: 3R, 1PR, 1CR (5/12cases).

Table (1): Oligonucleotide primer sequences used for amplification of the IgH and ß-globin genes.

| Primer | Primer sequence | Ref |
|---|---|---|
| FRIc | 5'-AGG TGC AGC TG (G/C) (A/T) G (G/C) AGT C (G/A/T) G G-3´ | 23 |
| FRIIa | 5´-TGG (A/G) TC CG (C/A) CAG (G/C) C (T/C) (T/C) CN GG-3´ | 24,25 |
| FRIIIa | 5´-ACA CGG C (C/T) (G/C) TGT ATT ACT GT-3´ | |
| LJH | 5´-TGA GGA GAC GGT GAC C-3´ | |
| VLJH | 5´-GTG ACC AGG GTN CCT TGG CCC CAG-3´ | |
| HBG7-F | 5´-GAAGAGCCAAGGACAGGTAC-3´ | 26 |
| HBG7-R | 5´-CAACTTCATCCACGTTCACC-3´ | |

Table (2): Detection rate of bone marrow involvement by biopsy in aspirate negative diffuse large B-cell lymphoma cases.

| Detection Method | Positive | Suspicious | Negative | Inadequate |
|---|---|---|---|---|
| Morphology | 8/41 (19.5%) | 11/41 (26.8%) | 16/41 (39%) | 6/41 (14.6%) |
| Immunohistochemistry | 3/41 (7.3%) | – | 8/41 (19.5%) | – |
| Total | 11/41 (26.8%) | 11/41 (26.8%) | 24/41 (58.5%) | 6/41 (14.6%) |

Table (3): Frequencies of amplified IgH framework regions in 41 DLBCL cases.

| FR | BM/PB | PB only | Single | Total |
|---|---|---|---|---|
| FRI | 10 | 1 | 11 | 21 |
| FRI, III | 7 | | 7 | |
| FRI, II, III | 2 | | 2 | |
| FRII | 2 | | 2 | 6 |
| FRIII | 2 | | 2 | 12 |
| FRI, FRII | 0 | 1 | 1 | |
| FRII, FRIII | 1 | | 1 | |

BMA : Bone marrow aspirate.
FR : Framework regions.
BM : And/or.
PB : Same FR patterns were detectable in peripheral blood and bone marrow of the cases.
(PB) : Rearrangements were detectable in peripheral blood only.

Table (4): Clinical staging vs. Staging by bone marrow biopsy morphology and IgH gene rearrangement.

| Clinical stage | No | Morphology | | | | Molecular | | Confirmed clinical stage: Negative by all methods |
|---|---|---|---|---|---|---|---|---|
| | | Inad | +ve | –ve | ? | +ve | % | |
| I | 12 | 2 | 2 | 5 | 3 | 7 | 58.3 | True stage I 4/19 (21%) |
| IE | 7 | 2 | 2 | 2 | 1 | 5 | 71.4 | |
| Total Stage I | 19 | 4 | 4 | 7 | 4 | 12 | 63.2 | |
| II | 9 | | 2 | 4 | 3 | 5 | 55.6 | True stage II 3/10 (30%) |
| IIE | 1 | | | | 1 | 0 | 0 | |
| Total stage II | 10 | | 2 | 4 | 4 | 5 | 50 | |
| III | 12 | 2 | 5 | 4 | 1 | 9 | 75 | True stage III 3/12 (25%) |

Inad: Inadequate sample ?: Suspicious

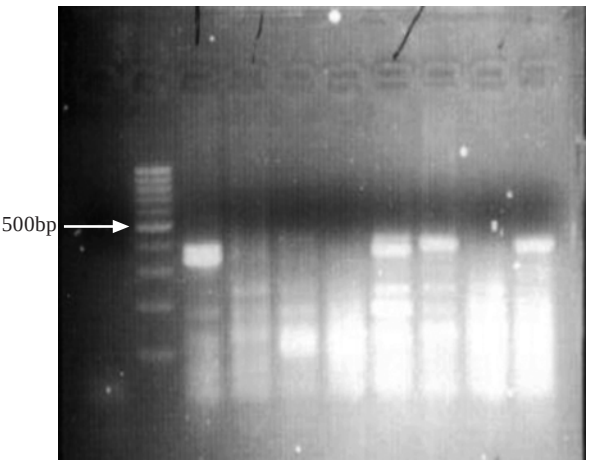


Fig. (1A): FRIc: Lane 1: Blank. Lane 2: 100 bp MWM, Lane 3: Positive control. Lane 4,5,6 and 9: Negative samples. Lane 7,8, and 10: One rearranged band 330-350 bp.

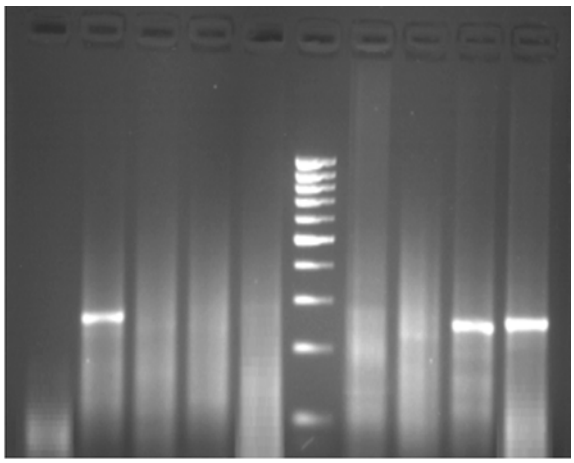

Fig. (1B): FRIIa: Lane 1: Blank. Lane 2: Positive control. Lane 3,4,5,7,8: Negative samples. Lane 6: 100 bp MWM. Lanes 9 and 10: One rearranged band 230-270 bp.

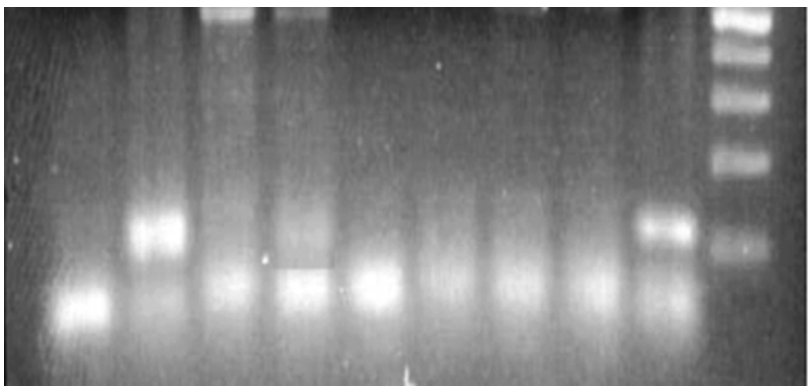

Fig. (1C): FRIIIa: Lane 1: Blank. Lane 2: Positive control. Lane 3,4,5,6,7,8: Negative samples. Lane 9: One rearranged band 70-110 bp. Lane 10: 100 bp MWM.

Fig. (1): Examples of PCR analysis of IgH gene rearrangements in BMA indicating the presence of clonally-rearranged cell population.

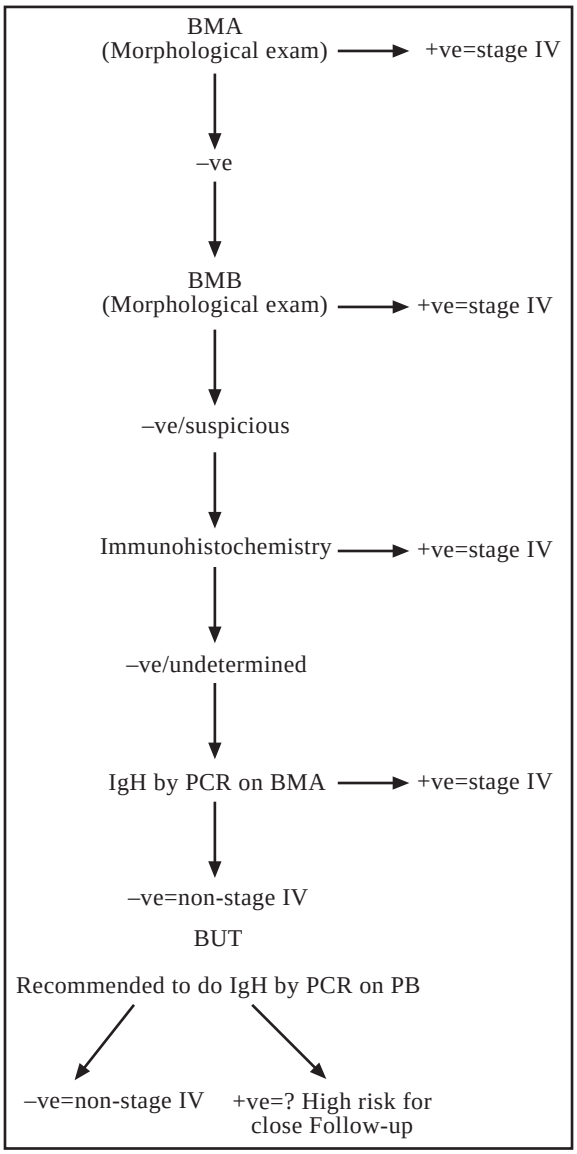


Fig. (2): Algorithm for detection of BM involvement in diffuse large B-cell lymphoma (DLBCL).

## DISCUSSION

Evaluation of bone marrow biopsies is part of the standard work-up for diagnosis, classification, and staging of non- Hodgkin's lymphoma (NHL). In addition, it is of major importance in monitoring the course of disease and in assessing the response to treatment. The appraisal of bone marrow involvement is traditionally based on morphological examination and has been shown to be prognostically relevant **[30]**. There have been several previous studies on the clinical role of ancillary investigations such as IHC and flow cytometry **[16]**, in NHL. For a long time, Southern blot analysis has been the gold standard technique for molecular clonality studies **[31]**. However, despite the high reliability of Southern blot analysis, it is increasingly replaced by PCR techniques. The immunoglobulin heavy chain (IgH) gene represents the most useful for detecting B-cell clonality since it rearranges early during B lymphoid development and demonstrates extensive junctional diversity. The incidence of informativity of IgH PCR in B-cell lymphomas varies with the IgH PCR strategy and the pathological subtype **[15,16,32]**.

A major goal of the current study was to define an algorithm for the diagnosis of bone marrow involvement in NHL patients with a high detection rate and the least possible cost and laboratory work-up.

By exclusion of the morphologically positive BMA cases, our detection rate by morphological evaluation of BMB was 19.5% of the BMA negative cases. This was lower than that observed in 2 studies (28.6% and 35% respectively **[10,33]** but higher than a third study, where morphologic evaluation of BMB was positive for involvement in 10.6% of the specimens **[34]**.

Combining the BMB with IHC, the detection rate increased to 26.8% and hence 11/41 cases were upstaged which is higher than other studies **[35,36]**. Also a study that used immunohistochemistry and immunophenotyping by flow cytometry in staging bone marrow biopsies reported an upstage of 20-22% of their patients with DLBCL **[16]**.

There are several previous studies addressing the role of gene rearrangement (IgH/IgL) in NHL with widely varying detection rates **[15,16,30,33,37,38]**. Using PCR amplification of IgH gene on DNA extracted from BMB, high detection rates ranging between 54.5% and 75% were reported **[30,33,38]** especially when using FRIII **[30]** or FRIII and FRII in a seminested PCR method **[33]**.

To increase the detection rate of the IgH PCR, 3 sets of consensus primers were used in this study including the commonly used FRIII. The consensus primers used were FRII-VH and, FRIII-VH specific primers. In addition the consensus FRIc primer directed against a highly conserved region in the FRI region shared by

the 7 VH families was employed for the FRI-JH amplification **[39]**. The higher detection rate observed with FRI in our study signified that FRI might be the primer with the highest sensitivity of detection of monoclonal IgH gene rearrangement in DLBCL cases. This result is supported by the finding of others **[23,40]** who used FRI primer in a predetermined algorithmic format. It has been reported that primers from FRI compared to the primers from FRIII, were more sensitive (73% vs 58%) in detection of monoclonality **[41]**.

PCR amplification of IgH gene detected clonality in 7/11, (63.6%) of our morphologically suspicious cases which was higher than a study **[30]** that reported clonality in 50% of morphologically suspicious DLCL cases. However, another study detected clonality in 3/3 of morphologically suspicious DLCL cases **[33]**. The number in this latter study is too few to make any conclusions. On the other hand, using FRIII and FRII in a single step PCR method did not detect any clonality among the 4 morphologically suspicious DLCLs **[42]**.

In this study, we detected clonality in 41.7% of the morphologically/IHC negative cases. Lower detection rates were previously reported. A lower detection rate 6.5% was reported **[42]** which might be explained by the use of FRIII and FRII in a single step PCR method. However, another study, using the biomed 2 protocol showed an even lower detection rate of only ~5% **[16]**.

One study reported a rate as high as 60% but the study involved a very small number (3/5 cases) **[33]**.

It is to be noted that, although our study was performed on BMA; it revealed a sensitivity of detection of monoclonality of IgH gene which was comparable to studies performed on BMB. This could be attributed to the contribution of the FRI primer which was successfully amplified from DNA extracted from BMA. Another contributing factor was the use of semi-nested PCR method which increases sensitivity besides decreasing the frequency of false negative results compared to the single step PCR method **[43]**.

In 2 cases clonality could be detected in PB but not in the corresponding BMA i.e testing PB for monoclonality of IgH improved the detection from 24/41 (58.5%) to 26/41 (63.4%). This finding of positive PB and negative BMA was previously reported **[15,44]**.

The 2 cases with PB positive for IgH clonality without corresponding positivity of BMA were morphologically suspicious (interstitial in one case and mixed paratrabecular interstitial in the other). One of them revealed B cell clonality by IHC analysis. A possible explanation for a false-negative result is that the lymphoma is present in the BMB but not in the BMA from which the DNA for our assay originated. Lymphomatous involvement of the BM tends to be focal, and it is entirely possible that the tumor was fortuitously present in the location where the biopsy needle was inserted, but was not present in the place from which the aspirate was taken. Moreover, the area of lymphomatous involvement, particularly paratrabecular sites, may be fibrotic and thus, not amenable to aspiration by vacuum suction **[45]**. This denotes that including PB in the test will overcome the limitation of using BMA instead of BMB for DNA preparation.

In this study, of the 41 stages I-III cases, only 10 (24.4%) were negative by both methods. Of the remaining 31, 26 (83.9%) showed IgH rearrangement and hence were actually stage IV. The remaining 5 cases were either suspicious or inadequate by morphology and were found negative by PCR.

Although there are reports that demonstrated a significant difference in overall survival at 5 years amongst patients with positive histology and molecular studies, negative histology but positive molecular studies, and negative histology and molecular studies **[15,46]**, others were unable to demonstrate such a difference or a change in the predictive value of the international prognostic index (IPI) by inclusion of molecular staging **[16]**. Similarly we could not draw any conclusions about the response to treatment amongst patients with positive or negative histology and molecular studies.

In Conclusion, our results confirm that PCR based IgH gene rearrangement analysis is a sensitive and specific method for demonstrating B cell clonality in BMA and PB. It should be considered as an additional tool to properly stage DLBCL cases according to the suggested algorithm.

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