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Clonality Assessment of B and T Cells: A Comprehensive Review of Techniques and Findings

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Abstract

For diagnosing a lymphoma by pathological examination, morphological assessment is the cornerstone, and based on the morphology of the cells, further ancillary tests like immunophenotyping and cytogenetics/ molecular studies are done to confirm the diagnosis. However, in 10-15% of non-Hodgkin lymphomas, morphology and immunophenotyping may not suffice for a definitive diagnosis, especially when no specific chromosomal translocations or other genetic abnormalities are known to be associated with that subtype of lymphoma. In these instances, assessing the clonality of lymphocytes is necessary to support the diagnostic suspicion of lymphoma. Various methods can be employed to determine the clonal nature of lymphocytes, including immunophenotyping techniques like flow cytometry and immunohistochemistry, as well as molecular approaches such as Southern blotting, Polymerase Chain Reaction (PCR) assays, and Next-Generation Sequencing (NGS). In addition to enhancing diagnostic accuracy, clonality assessment can also be valuable for evaluating minimal residual disease.

Keywords: T cell clonality; B cell clonality; Immunoglobulin gene rearrangement; TCR gene rearrangement.

Introduction

Morphology, immunophenotyping and identifying defined specific chromosomal translocations or genetic mutations are the cornerstone of diagnosing lymphomas. However, in 10-15% of non-Hodgkin lymphomas diagnosis is still a challenge [1], especially when a known specific translocation or mutation is not associated with the lymphoma. In instances when morphological distinction of lymphoma from a reactive process is ambiguous, it is necessary to do a clonality evaluation of lymphoid cells, as the malignant cells are monoclonal while reactive cells are polyclonal [2]. Clonality in a lymphoid cell population can be evaluated by multiple techniques. Initially, the identification of light chain restriction in B cell lymphomas through immunophenotyping was employed to ascertain clonality. Subsequently, immunophenotype-based tests, analogous to light chain limits, such as TCR V beta repertoire analysis and TRBC1 assays, have been implemented for the assessment of clonality in T cell lymphomas. Molecular diagnostic techniques such as Southern blotting, PCR, NGS can be employed to evaluate clonality through the characterisation of rearrangements in immunoglobulin and T-cell receptor genes.

B cells and T cells are integral components of the immune system, playing crucial roles in the recognition of foreign antigens. B and T cells possess hypervariable Immunoglobulin (Ig) molecules and T Cell Receptor (TCR) molecules respectively on their surfaces, which contribute to their remarkable diversity and ability to recognize a wide array of antigens. The Ig and TCR genes in germline DNA contain numerous Variable (V), Diverse (D), and Joining (J) segments that undergo random somatic rearrangement, resulting in the production of highly diverse Ig and TCR molecules.

B cells display an Ig molecule composed of a heavy chain (IGH) and one of two light chains, either Kappa (IGK) or Lambda (IGL) [3]. In contrast, TCR molecules on T cells are heterodimers that consist of either alpha and beta chains (αβ T cells) or gamma and delta chains (γδ T cells) [4]. The IGH, TCRβ, and TCRδ chains have V, D,

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and J segments within their variable regions, while the IGK, IGL, TCRα, and TCRγ chains include only V and J segments (Figure 1).

The diversity of B and T cells arises from several key processes. Unique V(D)J recombination occurs during B and T cell development, generating substantial diversity known as combinatorial diversity [5]. Additionally, variable numbers of nucleotides are added or deleted at the junctions of V/D/J segments, a phenomenon called junctional diversity, which further enhances the variability of the VDJ sequence [6]. Furthermore, in case of B cells, naive B cells in the germinal centre undergo somatic hypermutation upon exposure to antigens, resulting in changes to the VDJ sequence that increase the affinity of immunoglobulins for their target antigens [7]. Together, these three processes contribute to the generation of an incredibly vast repertoire of B and T cells, each with unique Ig and TCR sequences. The rearrangement of IG and TCR genes follows a hierarchical pattern: in IG genes, rearrangement begins with the IGH gene, followed by the IGK gene. If IGK rearrangement is non-productive, the IGK gene is deleted, and IGL gene rearrangement occurs. For TCR genes, rearrangement starts with the TCRD gene, followed by the TCRG gene, then the TCRB gene, and finally the TCRA gene [8].

Assessment of B-cell clonality by immunophenotyping

In B-cell non-Hodgkin lymphomas, clonality can be determined through light chain restriction within the malignant B cell population. Flow cytometry serves as a straightforward, cost-effective, and sensitive method for evaluating clonality via light chain restriction. In a typical polyclonal B cell population, the expected kappa to lambda light chain ratio ranges from 1:1 to 2:1 [9]. B cells from the same clone produce immunoglobulin that contains either kappa or lambda light chains [10] leading to an altered kappa to lambda ratio. Monoclonal B cells exhibit a kappa to lambda ratio of greater than 3:1 or less than 0.3:1 [11]. While the presence of normal B cells in a sample may partially normalize the kappa to lambda ratio, clonal B cells can be identified by their aberrant expression of B cell markers (Figure 2). Calculating the kappa to lambda ratio exclusively in the aberrant B cell population enables accurate determination of clonality, even in samples with a background of polyclonal B cells.

Light chain restriction can also be evaluated through Immunohistochemistry (IHC) and Chromogenic in Situ Hybridization (CISH). These methods have the advantage of being applicable to Formalin-Fixed Paraffin-Embedded (FFPE) tissue sections. Antibodies targeting kappa and lambda proteins are utilized to assess the expression of these proteins on B cell surfaces. However, IHC may present challenges, such as background staining from immunoglobulins in interstitial spaces or non-specific binding of antibodies to Fc receptors on antigen-presenting cells [12], which can affect the calculation of the kappa to lambda ratio. This limitation can be addressed by CISH, which employs probes to selectively stain kappa and lambda mRNAs within the B cell cytoplasm. Reports in the literature indicate considerable variability in the kappa to lambda ratios used to define clonal cells, ranging from more than 3:1 to less than 1:1, and more than 10:1 to less than 0.1:1. [13-17].

A notable limitation of using light chain restriction for detecting clonality is that rare biphenotypic B cell lymphomas having two sub-populations, one showing monoclonal expression of kappa and the other population showing expression of lambda light chain, is likely to mimic a reactive process and may be overlooked. Flow cytometry technique requires fresh sample and cannot be applied to FFPE sections. IHC may present challenges due to non-specific background staining, complicating the evaluation of the kappa to lambda ratio. In contrast, CISH can mitigate this issue but is a more complex technique that demands specialized technical expertise.

Several studies have demonstrated the utility of the immunoglobulin light chain ratio by flow cytometry, IHC and ISH in identifying clonal B cells in mature B cell neoplasms. Table 1 summarises findings and method of assessment of some of these studies. Chizuka et al. evaluated 105 cases of patients with persistently enlarged lymph nodes or extranodal tissues. They assessed the effectiveness of the light chain ratio in identifying clonal B cells and compared their findings with histopathological reports. The study found that the light chain ratio had a sensitivity of 73.1% and a specificity of 92.3% for identifying B cell lymphoma. Additionally, the positive Predictive Value (PPV) was 90.5%, and the negative Predictive Value (NPV) was 77.4% [18]. Mendes and Dreno compared IHC and In Situ Hybridization (ISH) methods to detect clonality in 38 cases of primary cutaneous B cell lymphoma. They found that IHC was not useful in 14 of the 38 cases, as these did not express monotypic immunoglobulins and were classified as non-secreting B cell lymphomas. However, ISH successfully demonstrated clonality in these cases. Based on these findings, the authors concluded that ISH could serve as a supplementary test to detect clonality when IHC is inconclusive or negative for light chain expression [19].

Paiva et al. conducted a study on the peripheral blood of 43 patients with mature B cell lymphoma, where they found that all 43 patients exhibited clonal B cells when analysing the light chain ratio by flow cytometry [20]. Guo et al. conducted a study on 203 tissue samples and 104 bone marrow aspirate samples from patients with suspected lymphoproliferative disorders, using both automated RNA In Situ Hybridization (RNA-ISH) and flow cytometry methods to assess the kappa lambda ratio for detecting clonal B cells and diagnosing mature B cell neoplasms. They found that RNA-ISH staining demonstrated light chain restriction with a sensitivity that was either equivalent to or greater than that of flow cytometry across a wide range of lymphoproliferative disorders. However, flow cytometry proved more effective than RNA-ISH staining for identifying clonality based on the kappa lambda ratio in bone marrow aspirate samples [21].

Shafeno et al. studied 45 cases of plasma cell myeloma and 45 cases of reactive plasmacytosis, using IHC to assess the utility of the kappa to lambda ratio in identifying clonal plasma cells in bone marrow biopsy sections. They found that IHC for the kappa to lambda ratio demonstrated a sensitivity of 100% and a specificity of 97.8% in detecting clonal plasma cells, making it a highly effective tool for diagnosing plasma cell neoplasms [22].

Assessment of T cell clonality

Identifying neoplastic T cells by flow cytometry poses challenges compared to B cell lymphomas, where clonality can be readily assessed through light chain restriction. In T cell malignancies, clonal T cells have traditionally been identified by immunophenotypic aberrancies, such as the under-expression, overexpression,

or absence of pan-T cell markers and the restriction of T cell subset antigens. However, these immunophenotypic alterations can also occur in reactive T cells [23]. Also, some neoplastic T cells may not exhibit obvious immunophenotypic aberrancies detectable by flow cytometry [24]. To address these limitations, two flow cytometric immunophenotypic methods have been developed: T-Cell Receptor Vβ repertoire (TCR Vβ-repertoire) analysis and T-cell Receptor β-chain Constant region 1 (TRBC1) expression. It is important to note that these techniques are applicable only to αβ T cells and not to γδ T cells. Table 2 summarises studies demonstrating the use of TCRVβ repertoire analysis and TRBC1 expression by flow cytometry to detect clonal T cells.

TCR Vβ repertoire analysis

In TCR Vβ repertoire analysis, the immunophenotypic assessment focuses on the variable regions of the TCR-β chain. Humans possess 65 genes coding for the variable region of the β chain, of which 47 are functional. These 65 genes can be categorized into 30 subfamilies based on sequence homology [25], with each subfamily containing one to nine members; a minimum of 75% sequence similarity is required for classification into a single subfamily. The IOTest Beta Mark TCR Repertoire Kit, supplied by Beckman Coulter, is a commercially available fluorescently tagged antibody kit that includes monoclonal antibodies targeting 24 TCR-Vβ subfamilies, representing approximately 70% of the normal human TCR-Vβ repertoire [26]. These antibodies are organized into eight cocktails, each containing three distinct subfamily-specific antibodies, with each cocktail containing one antibody labelled with Fluorescein Isothiocyanate (FITC), another with Phycoerythrin (PE), and the third labelled with both the fluorescent dyes. In a polyclonal T cell population, the 24 Vβ subfamilies are expected to be represented in a specific proportion out of all T lymhocytes. In contrast, an abnormal expansion of a single subfamily indicates T cell clonality. A sample is considered clonal if a single TCR-Vβ antigen is positive in more than 50% of the total analysed T cells or if it exceeds the expected normal maximum limit by tenfold (Figure 3). If a single TCR-Vβ antigen is positive in 40-49% of the total T cells, or if more than 70% of the total T cells are negative for all 24 antibodies, these findings suggest clonality. Samples that do not meet any of these criteria are classified as polyclonal [26,27].

Beck et al. studied 43 blood samples from patients suspected of having T-cell neoplasms. Of these, 27 samples were diagnosed as mature T-cell neoplasms based on clinical, morphological, immunophenotypic, and molecular findings. TCRVβ repertoire analysis was performed on all 48 samples to detect clonal T cells. The study found that TCRVβ repertoire analysis exhibited a sensitivity of 100% and a specificity of 88%, demonstrating its high accuracy in identifying clonal T cells in the diagnosis of mature T-cell neoplasms [28]. Tembhare et al. studied 31 peripheral blood samples, 3 bone marrow aspirates, and 1 lymph node aspirate from confirmed cases of mature T-cell neoplasm to detect clonal T cells using TCRVβ repertoire analysis. They were able to identify clonal T cells in all 41 cases. Additionally, the authors examined 61 Minimal Residual Disease (MRD) samples, including peripheral blood, bone marrow aspirates, and cerebrospinal fluid, from these 41 cases. They concluded that TCRVβ repertoire analysis is a valuable tool for detecting clonal T cells, even in low-cellularity samples, as long as the specific TCRVβ clone-specific single antibody cocktail is used for MRD assessment [26]. Salameire et al. studied 124 tissue biopsies from patients suspected of having mature T-cell lymphoma. Of these, 30 cases were diagnosed as mature T-cell neoplasms based on morphological, immunophenotypic, and molecular analyses. The authors used TCRVβ repertoire analysis to investigate the presence of clonal T cells in all 124 samples. They found that the technique had a sensitivity of 90% and a specificity of 98%, demonstrating its utility in detecting clonal T cells in suspected cases of mature T-cell lymphoma [29].

An additional advantage of this flow cytometry-based technique is its ability to directly quantify MRD, a capability not available with PCR-based methods. Although this flow cytometry assay is highly sensitive, it has limitations of high cost and it cannot be used to analyse clonality in γδ T cells. Furthermore, unlike PCR techniques, it cannot be employed for assessing clonality in fixed tissues. The requirement for eight tubes makes the assay cumbersome and labour-intensive, necessitating a sufficient sample volume to perform all eight tubes for clonality assessment.

Clonality assessment of T cells by TRBC1 (T-cell Receptor ß-chain Constant region 1)

A relatively simple, rapid, and low-cost method for assessing clonality in αβ-T cells is TRBC1 analysis by flow cytometry (Figure 4a). While analysis by TCR Vβ repertoire kit utilizes antibodies against the variable region of the β chain, TRBC1 analysis employs antibodies targeting the constant region of the β chain. The gene encoding the constant region of the β chain consists of two segments: constant region 1 (C1) and constant region 2 (C2). During T lineage commitment, when the β TCR undergoes rearrangement, either C1 or C2 is selected to code for the constant region. This selection is mutually exclusive, resulting in the β chain of any given T cell containing either C1 or C2. Hence, a normal healthy individual has two distinct populations of T cells in circulation i.e. TRBC1 expressing population and TRBC2 expressing population. There are commercially available antibodies specific to TRBC1, and the staining method for TRBC1 by flow cytometry has been standardized and validated, with established reference ranges for TRBC1 positivity in T cells [30]. Available data indicates that the average ratio of TRBC1-positive to TRBC1-negative cells is approximately 1:2 [31]. Cut-off values have been established, where a positivity of more than 85% or less than 15% for TRBC1, or a predominant population of T cells exhibiting dim positivity for TRBC1, is indicative of a clonal population [30,32].

However, certain considerations must be taken into account when analysing T cells for TRBC1 expression to define clonality. γδ T cells do not express TRBC1 because they lack a β chain; therefore, when evaluating CD8+ T cells or CD4/CD8 double-negative T cells for TRBC1 expression, it is crucial to exclude γδ T cells from the analysis before calculating the TRBC1 positive/negative population. Additionally, the TCR protein exists as a complex of CD3-TCR on the surface of T cells. Consequently, downregulation or aberrant absence of CD3 from mature T cell-surface can indicate a concurrent downregulation or absence of TRBC1. Thus, using TRBC1 to assess clonality in aberrant CD3-negative mature T cells or on immature T cells which normally do not express CD3 on surface, may lead to false positives for clonality [30].

Recently, an anti-TRBC2 antibody, which specifically binds to the TRBC2 antigen in the beta chain of αβ T cells, has been developed and recently made commercially available in some

countries, by Beckman Coulter, USA. When combined with anti-TRBC1 antibodies in a dual staining panel, this new antibody enables the study of T cell clonality by flow cytometry, similar to how kappa and lambda antibodies are used to assess clonality in B cells. Horna et al. conducted a study involving 60 samples of known T cell neoplasms, 104 clinical samples without T cell neoplasia, and 39 samples from healthy donors. Their findings confirmed the specificity of the anti-TRBC2 antibody for the TRBC2 antigen in the beta chain of αβ T cells. They also demonstrated that the dual staining method for TRBC1 and TRBC2 is especially valuable in cases with dim CD3 expression, where interpreting TRBC1 expression alone can be challenging. Furthermore, the study showed that cytoplasmic staining of TRBC1 and TRBC2 can be employed to identify clonal T cells in neoplasms lacking surface CD3 expression [33].

Berg et al. studied a total of 143 samples, including tissue and body fluid specimens, to investigate mature T-cell neoplasms. Of these, 46 samples were definitively diagnosed as mature T-cell neoplasms based on morphological, immunohistochemical, and molecular findings. The authors assessed TRBC1 expression in all the samples to detect clonal T cells. They found that all 46 mature T-cell neoplasm cases showed clonal T cells based on TRBC1 expression, while all 97 samples without T-cell neoplasms exhibited polytypic T cells, highlighting the technique's ability to distinguish between clonal and non-clonal T cell populations [32]. Capone et al. studied a total of 77 samples, including 14 from healthy donors as controls. Of these, 37 samples exhibited aberrant T cell populations by immunophenotyping. The clonality of these aberrant T cells was assessed using both TCRVβ repertoire analysis and TRBC1 expression. The results were concordant in 97% of the cases, with both techniques identifying clonal T cells. In one case, clonal T cells were detected by TRBC1 expression but not by TCRVβ repertoire analysis. However, molecular analysis confirmed the presence of clonal T cells in this sample, suggesting that TRBC1 expression analysis may be superior to TCRVβ repertoire analysis for detecting clonal T cells [24]. Nguyen et al. studied 90 cases of suspected T-cell neoplasm, excluding γδ T-cell neoplasms and mature T-cell neoplasms with dim or negative CD3 expression. Out of the 90 cases, 38 were confirmed as mature T-cell neoplasms based on morphological and immunophenotyping studies. The researchers assessed the presence of clonal T cells in all the samples using both TRBC1 expression and PCR-based methods. They found that 37 out of 38 mature T-cell neoplasm cases showed monotypic T cells by TRBC1 expression, while all 38 cases demonstrated monotypic T cells by PCR. The remaining 52 cases showed polytypic T cells by both TRBC1 expression and PCR techniques, indicating a non-clonal T-cell population [34].

Molecular methods for assessing clonality in B and T cells

Several molecular techniques can be utilized to evaluate clonality in B and T cells, including Southern blotting, Polymerase Chain Reaction (PCR)-based assays, and Next-Generation Sequencing (NGS).

Southern blot technique

Southern blot analysis was once considered the gold standard for assessing clonality in B and T cells but has largely been supplanted by PCR-based techniques [35]. In this method, DNA is fragmented using carefully selected restriction enzymes, and the resulting fragments are separated by size through gel electrophoresis before being transferred to a membrane. Radiolabelled DNA probes, designed to be complementary to immunoglobulin (Ig) and T-Cell Receptor (TCR) genes, are then applied to the membrane [36,37]. After washing away the unbound probes, the targeted fragments can be visualized using autoradiography. Distinct band patterns on gel for the normal polyclonal lymphocytes as compared to clonal lymphocytes help in assessment of clonality.

However, Southern blot analysis has several limitations, including requirement of fresh samples and a substantial amount of high-quality DNA (approximately 10,000 to 20,000 ng), having a long turnaround time, and need of technical expertise. Additionally, a minimum of 10-15% tumor cells in the sample is necessary to reliably identify clonality [38].

PCR-based methods

PCR has become the preferred method for assessing clonality, surpassing Southern blot techniques due to its speed, accuracy, and ability to work with very small amounts of DNA (50-100 ng). Moreover, PCR can be performed on Formalin-Fixed Paraffin-Embedded (FFPE) tissues, with sensitivity as low as 0.1% [35,39]. In 2003, the EuroClonality/BIOMED-2 consortium established standardized protocols and primers for multiplex PCR aimed at evaluating clonality in B and T cells, which has since been validated by numerous studies [40-44].

By 2012, EuroClonality/BIOMED-2 had published guidelines for the interpretation and reporting of Ig and TCR clonality [45]. PCR based method utilizes multiple consensus DNA primers that target conserved genetic regions within immunoglobulin chain genes and T cell receptor genes. A total of 14 multiplex PCR tubes have been designed by the EuroClonality/BIOMED-2 consortium for assessing clonality in Ig and TCR genes. These tubes include three for IGH (VH-JH) rearrangement, two for IGH (DH-JH), two for IGK (VK-JK, VK-Kde), one for IGL (VL-JL), three for TCRB (VB-JB, DB-JB), two for TCRG (VG-JG), and one for TCRD (VD-JD, VD, DD). In 2019, the consortium also published a single multiplex PCR tube assay specifically for TCRG genes [46]. Furthermore, the EuroClonality/BIOMED-2 consortium has recommended a stepwise algorithm for utilizing the multiplex PCR tubes to identify clonality [45]. In cases of suspected B cell neoplasms, the initial testing should involve the first three tubes for IGH gene rearrangement, ideally in conjunction with two tubes for IGK gene rearrangement. This approach is sufficient in over 95% of cases; however, if the initial results are inconclusive despite strong clinical suspicion of lymphoma, further testing with two IGH V-J tubes and an IGL tube may be warranted. For αβ T cell lymphomas, performing TCRB gene rearrangement studies alongside TCRG gene rearrangement studies—either in parallel or consecutively—is typically adequate. In contrast, for γδ T cell lymphomas, it is preferable to conduct TCRG gene rearrangement studies together with TCRD gene rearrangement studies.

Analysis of PCR products for clonality assessment

PCR product analysis can be performed using either gel-based assays or capillary electrophoresis. Both methods assess clonality based on the size of the PCR products.

Gel-based assays

In gel-based assays, heteroduplex analysis is employed, where the amplified PCR products are heat-denatured at 90°C and then rapidly cooled to 40°C for one hour [47]. This process allows the denatured DNA fragments to realign, forming heteroduplexes in cases of polyclonal PCR products, while monoclonal PCR products form homoduplexes. Heteroduplex molecules exhibit a slow-migrating smeared pattern on gel electrophoresis due to conformational differences, whereas homoduplexes appear as distinct single bands. In samples containing a polyclonal population amidst a monoclonal background, both homoduplexes and heteroduplexes may be observed [48]. This method is rapid, simple, cost-effective, and has a detection limit of 5% [35].

Capillary electrophoresis/GeneScanning

Capillary electrophoresis, or GeneScanning, involves fluorescently tagging single-stranded PCR products and separating them based on size in a capillary system. This method is preferred for clonality analysis due to its superior resolution (1-2 nucleotide differences), sensitivity of about 1%, and the automation that makes it rapid and less labour-intensive [49]. The EuroClonality/BIOMED-2 consortium published guidelines for interpreting and reporting PCR products analysed via GeneScanning in 2012 [45]. A normal polyclonal population typically displays a Gaussian distribution with multiple peaks, in contrast, a monoclonal population is characterized by one or two isolated peaks (Figure 4b), or one to two peaks that are at least twice as tall as the polyclonal peaks in the background [50]. The presence of two predominant peaks may indicate biclonality or biallelic rearrangement within a single clonal population. In the case of IGK and TCRB genes, multiple rearrangements can occur within a single allele, allowing for up to four peaks at the TCRB and IGK loci to still be consistent with a single clone. Therefore, interpreting multiple reproducible predominant peaks is complex, as it could signify biclonality, oligoclonality, or even pseudoclonality. The implications of such findings were also addressed in guidelines published by the EuroClonality/BIOMED-2 consortium in 2012 [8]. Occasionally, oligoclonal peaks identified in GeneScanning analysis may represent a pseudoclonal population, especially when selective amplification occurs from a sample containing a very small number of lymphoid cells [38,51]. However, in such cases, the predominant peaks are generally not reproducible, distinguishing them from true oligoclonal populations [45]. Additionally, since population discrimination relies on the size of the PCR products, false positives for clonality can arise when two or more clones share the same size. Nonetheless, the enhanced resolution of GeneScanning (1-2 nucleotide size differences) generally makes it more reliable than heteroduplex analysis.

False negatives for monoclonality may occur due to technical issues during PCR, such as improper primer annealing, which can result in incomplete amplification of the DNA fragments. Moreover, mature B cells that have undergone somatic hypermutation exhibit variations in the VDJ sequence of the IGH gene, which can hinder proper primer annealing, leading to potential false-negative results in B cell neoplasms of germinal centre or post-germinal centre origin [38,52].

Many studies (Table 3) have demonstrated the utility of PCR-based techniques to detect clonality by analysing IGH and TCR gene rearrangements are valuable tools in diagnosing B and T cell malignancies. Studies have also shown its usefulness in detecting MRD after treatment. Droese et al. studied 66 T-ALL samples and 36 mature T-cell neoplasm samples to detect clonal T cells using Southern blot and PCR techniques to study TCR gene rearrangement. In 91% of the analysed loci, the results from both methods were concordant. However, discordant results were observed in 9% of the cases. Specifically, 6% of loci showed monoclonal T cells by PCR but not by Southern blot, while 3% of loci showed monotypic T cells by Southern blot but not by PCR. These findings highlight that while both techniques are highly concordant, there can be occasional discrepancies in detecting clonal T cells, which may depend on the method used [53]. Melotti et al. studied 13 cases of Primary cutaneous B Cell lymphoma (PCBCL), 6 cases of pseudolymphomas, and 10 cases with inconclusive diagnoses by morphology and IHC to assess clonality in B cells using IGH gene rearrangement by PCR. Of the 13 confirmed PCBCL cases, 12 showed monoclonal B cells based on IGH and IGK gene rearrangement studies, while 1 case yielded a non-informative result for clonality. Among the 6 pseudolymphoma cases, 4 showed polyclonal B cells by PCR, and the remaining 2 cases had non-informative results. Of the 10 cases with inconclusive results by morphology and IHC, 2 demonstrated monoclonal B cells through IGH and IGK gene rearrangement analysis by PCR. The authors concluded that IGH and IGK gene rearrangement studies by PCR significantly improve the detection of clonality in cutaneous B cell lymphoma cases, especially in those with inconclusive diagnoses by traditional methods [54].

Xu et al. studied 25 cases of diagnosed mycosis fungoides, confirmed by morphology and immunophenotyping, as well as 6 cases of chronic inflammatory skin disease, to detect clonal T cells using PCR-based TCR gene rearrangement analysis. They found TCR gamma gene rearrangement in 23 of the mycosis fungoides cases and TCR beta gene rearrangement in 2 cases. One case exhibited both TCR gamma and TCR beta gene rearrangements. In contrast, none of the 6 chronic inflammatory skin disease cases showed TCR gene rearrangement, indicating the specificity of TCR gene rearrangement as a marker for clonal T cell populations in mycosis fungoides [55].

Ribera et al. studied 106 suspected cases of B and T cell lymphoma that showed inconclusive clonality by flow cytometry. They analysed IGH and TCR gamma gene rearrangements using PCR to detect clonality. Of the 106 cases, 36 showed clonal populations by PCR, with 27 of these confirmed as lymphoma through histopathological examination. Polyclonal results were obtained in 47 cases, 5 of which were later diagnosed as lymphoma by histopathology. Additionally, 14 cases had inconclusive clonality results by PCR, and 5 of these were subsequently confirmed as lymphoma. Finally, 8 cases showed non-informative results for clonality by PCR, 3 of which were later proven to be lymphoma. Based on these findings, the authors concluded that PCR is a valuable technique for defining clonality in cases where flow cytometry results are inconclusive [56].

Kavesh et al. studied the benefits of performing flow cytometry and PCR for IGH gene rearrangement in parallel to detect MRD in B lymphoblastic leukemia (B-ALL). A total of 119 cases were included in the study. The results showed that 85% of the cases exhibited concordant MRD detection by both flow cytometry and PCR, while 15% showed discordant results. Specifically, 12 cases

were positive for MRD by flow cytometry but negative by PCR, and 5 cases were positive for MRD by PCR but negative by flow cytometry. The authors noted that all 5 cases with discordant results, where flow cytometry was negative for MRD, had dim CD10 expression, and one patient had also undergone Chimeric Antigen Receptor (CAR) T-cell therapy. This could have made it more challenging to detect MRD by flow cytometry. The authors hypothesized that heavy somatic hypermutation in conserved regions of the IGH gene and other technical limitations with the PCR technique might explain the discordance in these cases. Based on their findings, Kavesh et al. concluded that IGH gene rearrangement studies could be particularly useful for detecting MRD in B-ALL cases with dim CD10 expression or in patients who have undergone CAR T-cell therapy, where flow cytometry might fail to detect MRD [57].

Clonality assessment by Next Generation Sequencing (NGS)

Next Generation Sequencing (NGS), also known as massive parallel sequencing, is a powerful technology that allows for the simultaneous sequencing of millions of DNA segments, providing high throughput and detailed insights into genetic rearrangements. Unlike PCR-based clonality assays, which rely on the size of PCR amplicons to assess clonality, NGS evaluates the specific sequences of IG and TCR segments, offering a more comprehensive approach to clonality assessment [58].

Development of NGS-based clonality analysis

In 2019, the EuroClonality/BIOMED-2 consortium introduced NGS-based clonality analysis specifically targeting IG gene rearrangements [59]. Alongside this development, they created a web-based interactive bioinformatics application called ARResT/Interrogate, which analyses sequence data for Variable (V), Diversity (D), and Joining (J) genes to assign clonality based on the sequence characteristics [60]. This protocol underwent validation in a multicentre study, demonstrating impressive interlaboratory concordance (99%) and a high level of agreement with conventional clonality analysis (98%) [61].

Further validations of NGS-based IG and TCR gene rearrangement assays have confirmed their reliability, showcasing high concordance rates with traditional methods [62,63]. NGS assays are particularly advantageous when analysing suboptimal quality samples, such as those derived from Formalin-Fixed Paraffin-Embedded (FFPE) tissues, where shorter DNA fragments can still be effectively analysed [64].

Advantages in minimal residual disease detection

One significant advantage of NGS-based assays over conventional Capillary Electrophoresis (CE) assays is their ability to detect small clonal populations that may be obscured within a larger polyclonal background. This capability is crucial for MRD detection, as NGS can identify tiny populations of clonal cells based on the specific sequences of the VDJ segments, outperforming traditional CE-based assays in this aspect [64-66].

Disadvantages and challenges

Despite the advancements that NGS technology brings, several challenges remain. A key limitation is the lack of uniformity in the interpretation and reporting of NGS results. Cut-off values for determining whether a readout is considered clonal vary significantly across different publications [62,63,67,68]. This inconsistency can lead to discrepancies in clinical practice and interpretation, necessitating the development of standardized guidelines for the broader adoption of NGS in clinical settings. In conclusion, NGS represents a robust and versatile approach for assessing clonality in B and T cells, with notable advantages in sensitivity and the ability to analyse low-quality samples. However, the field must address issues of standardization in interpretation to fully realize the potential of NGS in clinical diagnostics.

Several articles highlight the advantages of NGS-based methods for studying IGH and TCR gene rearrangements compared to traditional techniques like PCR, emphasizing the superior sensitivity, specificity, and ability of NGS to analyse a broader spectrum of clonal rearrangements (Table 4). Kirsch et al. studied 39 samples of confirmed cutaneous T cell lymphoma to detect clonal T cells by analysing TCR beta and TCR gamma rearrangements using both NGS-based and PCR-based techniques. The study found that all 39 samples showed clonal T cells by the NGS-based method, while the PCR-based technique identified clonal T cells in only 27 out of 39 samples (70%). The results demonstrated that the NGS-based technique was superior to the PCR-based method in detecting clonality in T cell neoplasms [69]. Arcila et al. studied a total of 716 samples, including those from acute B and T leukemia, mature B and T cell lymphomas, and plasma cell neoplasms. The cohort included 534 samples taken at diagnosis and 182 follow-up samples for disease monitoring. At the time of diagnosis, 94% of cases showed clonality by NGS, compared to 89% detected by PCR. In post-therapy follow-up samples, NGS identified MRD in 25 more cases than flow cytometry (137/182 cases vs. 112/182 cases). The MRD detected by NGS was further confirmed by PCR. The sensitivity of MRD detection by NGS was 10^{-5}, while flow cytometry sensitivity ranged from 10^{-4} to 10^{-5}. The authors concluded that NGS-based methods for assessing clonality in B and T cell neoplasms demonstrated a significantly improved detection rate compared to PCR. Additionally, MRD detection by NGS showed superior sensitivity and a higher detection rate than flow cytometry [70]. Kansal et al. studied 41 archived cases, including both lymphoproliferative disorders (LPD) and cases with no history of LPD, to assess clonal T cells by studying TCR gamma rearrangement using both PCR-based and NGS-based methods. The study found that NGS detected clonality in 8 cases (19% of the cohort) where the PCR-based method failed to identify clonal populations. The authors concluded that NGS demonstrated significantly higher sensitivity compared to PCR-based techniques and was capable of detecting small clones, including both monoallelic and biallelic clones, within all T cells [71]. Svaton et al. compared MRD detection using PCR-based techniques and NGS based methods in a cohort of 432 children with B-ALL, for whom IG/TCR MRD markers were identified at the time of diagnosis. The study analysed 780 IG and TCR markers in bone marrow samples collected on day 33 post-therapy, using both PCR and NGS-based methods. The results showed that 81.9% of the markers yielded concordant results between the two techniques. However, 4.7% of markers were detected by NGS but not by PCR, while 13.3% of markers were identified by PCR but not by NGS. Upon further analysis, the authors found that the IG/TCR rearrangements detected by PCR but not by NGS were also identified in unrelated samples, leading them to classify these as non-specific or false positives. Based on

these findings, the authors concluded that while NGS-based MRD detection was highly concordant with PCR-based detection, it was more specific and eliminated false-positive MRD results, offering a more reliable method for MRD assessment in B-ALL [72].

Clonal B or T cells in benign conditions

When interpreting results from clonality assays, it is crucial to consider the clinical context, morphology, and immunophenotype of the cells or tissue under examination. Monoclonal B or T cells can occur in benign conditions, which may lead to potential misinterpretations of clonality. In particular, in immunocompromised patients with a limited repertoire of epitopes, the selection pressure may favor the emergence of dominant clones due to chronic antigen stimulation [38,51]. Additionally, in T cell-mediated autoimmune diseases, it is common for autoreactive T cells to undergo clonal activation and subsequent expansion. Furthermore, studies indicate that clonal T cell expansions can be observed in at least 55% of healthy individuals over the age of 65, suggesting that age-related factors may contribute to such phenomena [38,73].

IG/TCR gene re-arrangements and their implications in lineage assignment

It is important to note that IG and TCR gene rearrangements are not strictly lineage-specific. These rearrangements can occur across various hematological malignancies, including B cell lymphomas, T cell lymphomas, and even in conditions like Acute Myeloid Leukemia (AML) [74-77]. For instance, B cell lymphomas may present with IG gene rearrangements, with or without TCR gene rearrangement. Conversely, T cell lymphomas can also exhibit IG gene rearrangements, illustrating the complexity and overlap between different lymphoid neoplasms [74].

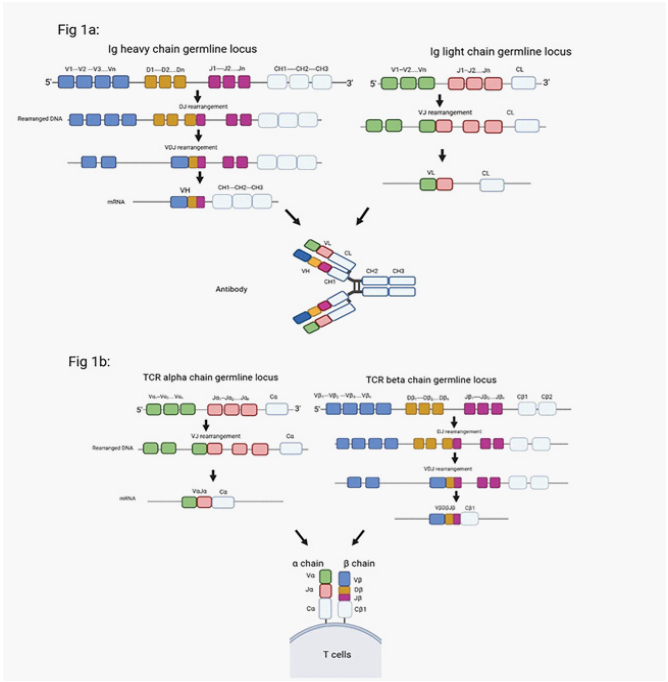

Figure 1: (A) A schematic illustration of the V(D)J recombination process for generating a V(D)J exon. During this process, one V gene segment, one D gene segment (if present in the gene locus), and one J gene segment are randomly chosen and rearranged to create a V(D)J exon. This rearranged exon encodes the variable region of the antigen receptor in B lymphocytes. **(B)** A Schematic representation of V(D)J recombination for generation of V(D)J exon that encodes variable region in alpha and beta chains of T cell receptor.

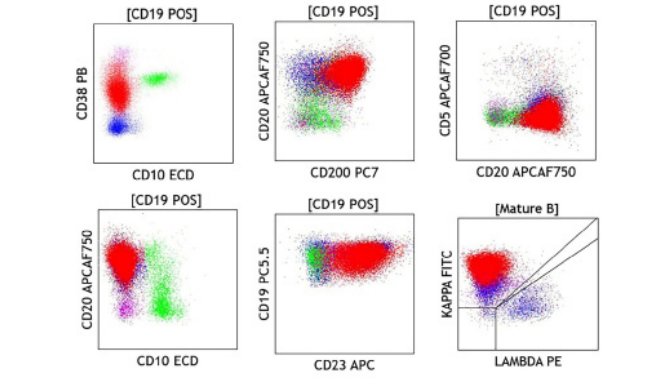

Figure 2: Flow cytometry plots showing abnormal clonal B cells (red population) that are CD38 positive, CD19 positive, CD20 positive, CD200 positive, CD23 heterogenous positive, and kappa restricted. (Blue population- normal B cells, green population-hematogones).

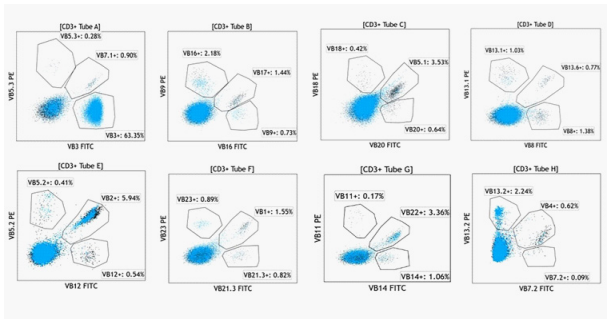

Figure 3: Flow cytometry plots showing TCR VB repertoire analysis with representative plots from eight tubes (Tube A to Tube H). Tube A showing 63.4% cells expressing VB3 antigen indicating clonality. These cells are CD8 restricted. (Blue population- CD8 positive T cells, Black population- CD4 positive T cells).

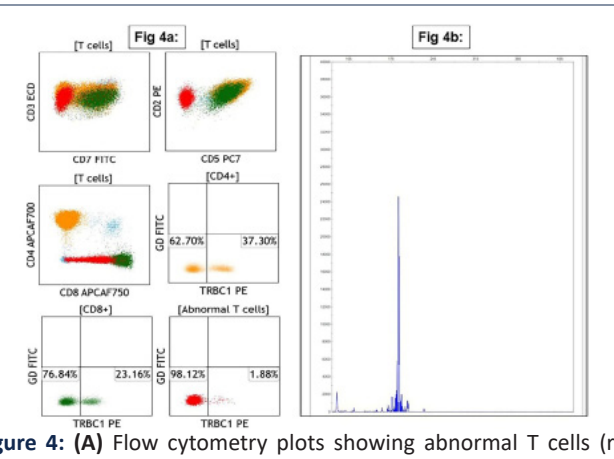

Figure 4: (A) Flow cytometry plots showing abnormal T cells (red population) that are CD8 dim and showing loss of CD7 & CD5. These cells are TRBC1 negative, indicating clonality. **(B)** TCR gamma gene clonality assessment by PCR method, genescan of the PCR products showing single tall peak that is more than 2 times that of the tallest peak in the background indicating clonality.

Table 1: Summary of studies on the utility of immunoglobulin light chain ratio in identifying clonal B cells.

Study	Number of samples	Method of clonality assessment	Key points
Chizuka et al., 2002	105	Flow cytometry	Sensitivity-73.1%, Specificity-92.3%, PPV-90.5%, NPV-77.4%
Mendes and Dreno et al., 2003	38	IHC and ISH	ISH serves as a supplementary test to detect clonality when IHC is inconclusive or when the clonal B cells are non-secretory cells
Paiva et al., 2018	43	Flow cytometry	100% of B cell lymphoma cases demonstrated clonality by K:L ratio assessment by flow cytometry
Gio et al., 2018	307	ISH and flow cytometry	ISH has better sensitivity than flow cytometry to demonstrate clonality in tissue samples and flow cytometry is more effective in demonstrating clonality than ISH in bone marrow aspirate samples.
Shafeno et al, 2019	90	IHC	IHC in bone marrow biopsy specimen of plasma cell neoplasm has a sensitivity of 100% and specificity of 97.8% in detecting clonal plasma cells

Table 2: Summary of studies demonstrating the use of TCRVβ repertoire analysis and TRBC1 expression by flow cytometry to detect clonal T cells:

Study	Number of samples	Method of clonality assessment	Key points
Beck et al., 2003	43 blood samples	TCRVβ repertoire analysis	Sensitivity-100%, Specificity- 88%
Tembhare et al., 2011	41 samples at time of diagnosis, 61 post therapy follow up samples including peripheral blood, bone marrow, tissues and body fluids	TCRVβ repertoire analysis	All 41 samples at time of diagnosis demonstrated T cell clonality. TCRVβ repertoire analysis is a valuable tool in identifying MRD even in low cellularity samples like body fluids.
Salameire et al., 2012	124 tissue biopsies (30 of these were diagnosed as mature T cell neoplasm by further investigations)	TCRVβ repertoire analysis	Sensitivity-90%, specificity-98%
Berg et al., 2021	143 samples including tissues and body fluids	TRBC1 expression	All 46 T cell neoplasms demonstrated clonality with TRBC1 expression and all 97 samples without T cell neoplasia demonstrated polytypia of T cells.
Capone et al., 2022	77 peripheral blood and bone marrow aspirate samples (37 cases were diagnosed to be T cell neoplasia and 14 samples were from healthy donors)	TRBC1 expression Vs TCRVβ repertoire analysis	97% concordance between both the methods. One case showed clonality by TRBC1 expression but not by TCRVβ repertoire analysis which was confirmed by molecular methods.
Nguyen et al., 2024	90 peripheral blood, bone marrow and lymph node samples (38 of which were diagnosed to be mature T cell neoplasm by further studies)	TRBC1 expression Vs PCR based methods to detect clonality in T cells	37 out of 38 cases showed clonal T cells by TRCB1 expression while all 38 cases showed clonal T cells by PCR method. All 52 cases demonstrated polytypia by both TRBC1 expression and PCR method.

Table 3: Summary of studies demonstrating the use of PCR based methods to detect clonal B or T cells.

Study	Number of samples	Method of clonality assessment	Key points
Droese et al., 2004	66 peripheral blood and bone marrow aspirate samples (including T-ALL samples and 36 mature T cell neoplasm samples)	Southern blot versus PCR (both Gel electrophoresis and Capillary electrophoresis) to detect TCR gamma rearrangement	91% concordance between both the methods, 9% showed clonality by only PCR methods and 3% showed clonality with only Southern blot method.
Melotti et al., 2010	29 skin biopsy samples (includes 13 cutaneous B cell lymphoma samples, 6 pseudolymphomas and 10 cases with inconclusive diagnosis)	PCR based IGH and IGK gene rearrangement by Gel electrophoresis.	12 out of 13 B cell lymphoma cases demonstrated clonal B cells. 4 out of 6 pseudolymphoma demonstrated polyclonal B cells, rest 2 showed non-informative results. 2 out of the 10 cases with inconclusive biopsy report demonstrated clonal B cells.
Xu et al., 2011	31 skin biopsy samples (25 diagnosed cases of mycosis fungoides (MF) and 6 chronic inflammatory skin disease samples)	TCR beta gene and TCR gamma gene rearrangement by Gel electrophoresis.	All 25 cases of MF showed clonal T cells while all 6 samples with chronic inflammation showed polytypic T cells.
Ribera et al., 2013	106 peripheral blood, bone marrow or lymph node samples of suspected B or T cell lymphoma with inconclusive flow cytometry reports.	IGH and TCR gene rearrangement study by PCR followed by capillary electrophoresis.	36 out of 106 samples showed clonal B or T cells, 27 of these confirmed to be lymphoma by further studies. 47 out of 106 samples showed polyclonal B or T cells, 5 of which turned out to be lymphoma by further studies.
Kavesh et al., 2020	119 bone marrow aspirates from B-ALL post therapy samples	Flow cytometry by immunophenotyping versus PCR-Capillary electrophoresis to detect MRD by IGH gene rearrangement study.	85% concordance between 2 methods. 12 samples showed positive MRD by FCM but negative by PCR. 5 samples (had dim CD10 expression and one was post CART sample) showed negative MRD by FCM but positive MRD by PCR.

Table 4: Summary of studies demonstrating the use of NGS based methods to detect clonal B or T cells:

Study	Number of samples	Method of clonality assessment	Key points
Kirsch et al., 2015	39 skin biopsy samples (confirmed cases of cutaneous T cell lymphoma)	NGS versus PCR-Capillary electrophoresis.	All 39 samples demonstrated clonal T cells by NGS method and only 70% of samples showed clonal T cells by PCR method.
Arcila et al., 2017	716 peripheral blood or bone marrow samples (includes B and T acute leukemias, B and T mature lymphomas and Plasma cell neoplasms). 534 samples were at time of diagnosis and 182 samples were post therapy follow up samples	NGS versus PCR-Capillary electrophoresis.	94% concordance between both the methods in case samples at baseline. NGS detected clonality in 94% of samples versus 89% in case of PCR. NGS detected MRD in 25 more cases (137 Vs 112) compared to PCR method.
Kansal et al., 2018	41 tissue biopsies (including 20 cases confirmed to be mature T cell neoplasm and 21 cases without a diagnosis of T cell neoplasm)	NGS versus PCR-Capillary electrophoresis.	PCR method detected 19% less cases with clonal T cells compared to NGS based method.
Svaton et al., 2023	432 B-ALL post therapy bone marrow aspirate samples	NGS versus PCR-Capillary electrophoresis.	High concordance between both the methods. NGS based method was more reliable as PCR based method showed false positivity in 13.3% markers studied.

Conclusion

In summary, clonality assessments in B and T cell lymphomas are essential diagnostic tools, particularly in 10-15% of NHLs where morphology and immunophenotyping alone are insufficient to distinguish between reactive processes and true malignancies. Among the various methods available for assessing clonality, immunophenotyping is relatively straightforward but often requires corroborative molecular assays for definitive results. Although Southern blotting was once the gold standard for assessing clonality, it has largely been supplanted by PCR-based assays due to their efficiency and reliability. More recently, NGS has emerged as a promising tool for clonality assessment, albeit with challenges in standardization and reporting. Each method for assessing clonality has its own set of advantages and disadvantages, and thus, it is imperative to utilize these techniques in a complementary manner rather than favouring one method over another. By integrating multiple approaches, clinicians can achieve a more accurate and comprehensive assessment of clonality in B and T cell lymphomas, leading to improved diagnostic accuracy and patient management.

Declarations

Conflicts of interest/competing interests: The authors declare that they have no conflict of interest.

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