PCR analysis of immunoglobulin heavy chain and TCR gene rearrangements in diagnosis of lymphoproliferative disorders on formalin-fixed, paraffin-embedded tissues

M. SVACHOVA* , M. TICHY

Laboratory of Molecular Pathology and Department of Pathology, Faculty of Medicine and Dentistry, Palacky University, Hnevotinska 3, 775 15 Olomouc, Czech Republic; e-mail: msvach@seznam.cz

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To confirm a diagnosis of malignant lymphomas it is imperative to distinguish between reactive and neoplastic proliferation. The PCR (polymerase chain reaction) is a method that can be used for detection of clonal rearrangements of the immunoglobulin heavy chain (IgH) and T-cell receptor (TCR) genes. This study summarizes the outcomes of PCR analysis of IgH and TCR gene rearrangements in 91 bioptic cases of lymphoproliferative disorders. In the class of B lymphomas we detected clonal IgH rearrangement in nearly 83% of cases and in class of T lymphomas in 81% of cases. We can affirm that PCR analysis of B and T cell clonality on DNA extracted from the whole section of formalin-fixed, paraffin-embedded tissue is very suitable for routinely elaborate this. Its influence on the diagnostics of morphological unclear cases in particular, is crucial and is useful in establishing a diagnosis of lymphoid neoplasias in specimens in which histological and immunophenotypic studies are inconclusive.

Key words: malignant lymphoma, clonality gene rearrangement, polymerase chain reaction

Malignant lymphomas are neoplasms that arise from lymphoid cells of either B-cell or T-cell lineage at various stages in normal lymphocyte development [1]. They can be very difficult to distinguish reliable with routine histopathological methods only. The establishment of monoclonality in lymphoproliferative processes has, therefore, become an important adjunct in the diagnosis of malignant lymphoma. Polymerase chain reaction (PCR) assays are able to demonstrate clonal IgH gene and TCR gene rearrangement in lymphoid malignancies using DNA extracted from formalin-fixed, paraffin embedded tissue (FFPE). The sensitivity and reproducibility of the PCR method can be negatively influenced by small quantites of often poor quality DNA [2-4] due to damage caused by formalin fixation, such as cross-linking between nucleic acid strands, DNA adducts with histones and base modifications [5, 6].

To generate enormous numbers of variable B- and T- cell antigen receptors, the immunoglobulin (Ig) and T-cell receptor (TCR) genes are somatically assembled from variable (V), diversity (D) and joining (J) gene segments [7-11]. This process of somatic recombination called DNA rearrangement involves recognition and double-stranded cleavage by RAG1 and RAG2 (recombinase-activating gene 1 and 2) protein complexes which recognize highly conserved RSSs (recombination signal sequences) flanking Ig and TCR gene segments [7-9, 12, 13]. The result is a ligated coding joint that forms the functional gene product. The sequences between rearranged gene segments are generally deleted in the form of a circular excision product, also called a TCR excision circle (TREC) or B-cell receptor excision circle [8, 10]. The variable-diversity-joining region (V(D)J) recombination is essential for functional expression of Ig and TCR genes and is critical for generating antigen recognition diversity in the immune system [12, 14, 15]. Further variability is introduced by insertion of N-nucleotides by TdT (terminal deoxynucleotidyltransferase) at the V-J and D-J junctions; the sequence and the length of insertion are random [1, 3, 16-20]. Other mechanism contributing to this variability includes somatic hypermutation which is restricted to the later, germinal center phase of B cell development [21, 22].

* Corresponding author

Such rearrangements are widely used as markers of B and T cell clonality and can distinguish a reactive (polyclonal) from a neoplastic (monoclonal) proliferation [21, 23-25]. In reactive proliferation, there are many different clones which have different rearrangements of their genes and the PCR amplifies DNA segments ranging in size depending on N-nucleotides insertion by TdT. This normal distribution due to N-region insertion and to the exonuclease activity of the recombinase system is seen as a smear on agarose gel. By contrast, the neoplastic cells in a monoclonal proliferation produce a PCR product of uniform size (fragments of homogeneous length) [11, 22] and after electrophoresis, one or two distinct bands are determined depending on whether one or both alleles were rearranged [3].

Materials and methods

Patients and Specimens Studied. All of the clinical samples studied were obtained from formalin-fixed, paraffin-embedded tissue biopsy selected from the files of the Department of Pathology at the University Hospital in Olomouc and from bioptic material delivered to our department for the "second reading" from other hospitals. We began using the PCR methodology for diagnostic purposes in our laboratory in 2003. This report includes 91 samples which were used to increase the number of well-characterized neoplastic and non-neoplastic lesions and to perform clinicopathologic correlations. We investigated 55 malignant lymphomas (29 from B-cells and 26 from T-cells) and 36 various nodal and extranodal non-neoplastic lesions (from skin, nasopharynx, duodenum, lung, bone marrow, tonsils, mamma, conjunctiva, rectum). The surgical and pathologists diagnosis were unknown to the person performing the PCR analysis.

DNA extraction. Five paraffin sections 5 μm thick from each sample were placed in screw-cup Eppendorf tubes. 300 μl of xylene was added to dissolve the paraffin and was incubated 15 minutes with constant gentle mixing at room temperature, then centrifuged at 13,400 x g for 5 minutes to pellet the tissue. The supernatant was removed, the dewaxing was repeated twice and 300 μl absolute ethanol was added. After vortexing and centrifuging for 3 minutes, the ethanol was pippetted off. This ethanol washing procedure was repeated once more.

The Puregene® DNA isolation Kit (Gentra Systems, Minneapolis, MN) was used to isolate the genomic DNA. Then we added 300 μl of Cell Lysis Solution with 1.5 μl Proteinase K Solution (20 mg/ml) (Gentra Systems, Minneapolis, MN) and incubated it at 56°C overnight. The cell lysate was treated by the addition of 1.5 μl RNase A Solution (4 mg/ml) (Gentra Systems, Minneapolis, MN) and incubated at 37°C for 45 minutes. The samples were then cooled to room temperature and 100 μl of Protein Precipitation Solution was added followed by vortexing the tubes vigorously at high speed and centrifuging at 13,400 x g for 3 minutes. The precipitated proteins formed a tight pellet. The supernatant containing the DNA was poured into a clean 1.5 ml microfuge tubes containing 300 μl of 100% isopropanol. The tubes were gently inverted several times and centrifuged for 5 minutes. The supernatant was poured off and 300 μl of 70% ethanol was added to wash the pellet. After centrifuging for 1 minute and carefully pouring off the ethanol, the tubes were drained. Then 20 μl of DNA Hydration Solution was added and DNA was rehydrated by incubating samples for 1 hour at 65°C. The tubes were periodically tapped to aid in dispersing the DNA. After isolation, DNA yield and quality was confirmed by spectrophotometric analysis.

Primers and PCR Conditions. The PCR is a method currently used for detecting IgH and TCR gene rearrangements. In the PCR analysis, DNA is amplified with a series of consensus primer pairs (GENERI BIOTECH, CZ) that bind to sequences of variable, diversity, and joining regions of these genes. TCR gene rearrangement is more difficult to demonstrate than the IgH gene by reason of relatively highly conserved target regions at two frameworks of rearranging IgH genes which are amplificated. In contrast, the genomic organisation of TCR genes is highly complex [2, 26]. In our laboratory, analysis of TCR gene rearrangements was performed using multiplex PCR with sets of primers Vγ11-Jγ11 for TCRγ that produce bands ranging between 70 and 110 bp [17, 27] and Tβ V-Tβ J1, Tβ D1-Tβ J1, Tβ D1-Tβ J2, Tβ D2-Tβ J2 for TCRβ. The PCR products range from 55 bp to 100 bp [16, 27]. The pair of primers CF20i 5'-CF20i 3' was used to verify amplification of the individual samples and amplify a region approx 460-500 bp in exon 20 of the cystic fibrosis gene. This amplification product serves as an internal control (housekeeping gene). The variable region of the IgH gene is composed of a large number of gene segments, each of which contains three hypervariable complementary determining regions (CDR I-III) and three relatively conserved framework regions (FR I-III) [28, 29]. The rearrangements of the IgH gene were evaluated using two seminested PCR systems [11, 28-32] and two multiplex PCR systems. In FR2A/LJH-VLJH system, the primers are homologous to the conserved sequences of the second framework of the V-region; expected band size is 240-260 bp, and the FR3/JLH-VLJH primers are homologous to the third framework of the V-region; expected band size is 100-120 bp. Both of these seminested PCR systems employ an external LJH and internal VLJH primer homologous to the consensus sequences of the J-regions of the IgH gene [29, 33]. The multiplex PCR assays for the IgH chain gene employ VH primers. In our laboratory, we used the JH1-S primer that binds to the framework III region; the PCR products range from 100 to 150 bp and JH2-S primer that binds to the framework II region that produce a band ranging between 220 and 250 bp. The downstream primer JH-A anneals to one of the J segments [27]. Usually a single J region primer is sufficient to recognize all six possible JH segments but no single V region primer recognizes all V segments, since there are many more V segments and they are more heterogenous than the J segment [19]. The primer sequences used

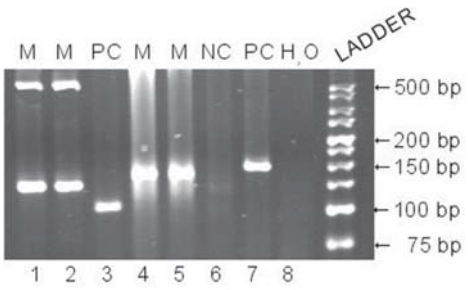

Figure 1. Examples of PCR analysis of IgH gene rearrangements using JH1-S/JH-A pair of primers. Agarose gel (3%) stained with ethidium bromid. Lane 1 and 2, extranodal marginal zone B-cell lymphoma; lane 3, monoclonal control (B-cell lymphoma); lane 4 and 5, folicullar lymphoma; lane 6, polyclonal control (reactive lymph node); lane 7, monoclonal control (B-cell lymhoma); lane 8, negative control (H_2O). M-monoclonal proliferation, PC-positive control, NC-negative control.

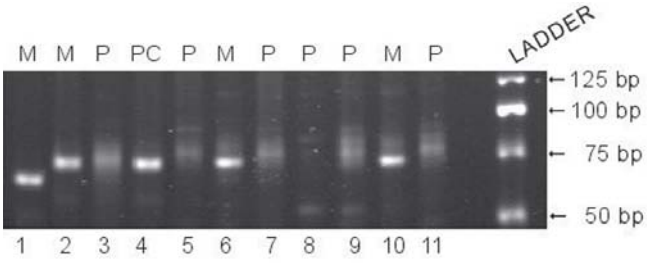

Figure 2. Examples of PCR analysis of TCR gene rearrangements using Vγ11-Jγ11 (lane 1-4); Tβ D1-Tβ J2 (lane 5-7); Tβ D2-Tβ J2 (lane 8-11) pair of primers. Agarose gel (3%) stained with ethidium bromid. Lane 1, subcutaneous panniculitis-like T-cell lymphoma; lane 2, 6,10, peripheral T-cell lymphomas, unspecified; lane 3, polyclonal control (reactive lymph node); lane 4, monoclonal control (T-cell lymphoma); lane 5, 7-9, 11, polyclonal smear. M-monoclonal proliferation, P-polyclonal proliferation, PC-positive control, NC-negative control.

[27, 34] are given in Table 1. To reduce false negative results, amplification of housekeeping genes was performed on cases of the rearrangement of the IgH and TCR genes as well. Cases that were not amplified by these housekeeping genes indicated that the DNA extracted was not suitable for PCR. DNA samples from B and T lymphoma cases with well-established clonal rearrangement were employed as monoclonal controls. Water was included as a negative control in all reactions to monitor contamination of samples.

Polymerase chain reaction. To improve the sensitivity of clonal analysis, we used a heat-activated thermostable DNA polymerase providing both 5'→3' polymerase and 3'→5' exonuclease activities which can eliminate the production of non-specific reaction products such as primer-dimers and misprimed products [21]. The reaction mix (25 µl final volume) consisted of template DNA (50-500 ng), 10x ImmoBuffer and 50 mM $MgCl_2$ Solution (Bioline, USA Inc.), 100 mM dNTP Mix (Promega Madison, Wi, USA), 0.1 mM of each primer, 1 U IMMOLASE™ DNA Polymerase (Bioline, USA Inc.) and distilled water.

The PCR programme (for multiplex PCR analysis of TCR and IgH, and for the first round of seminested PCR analysis of IgH) consisted of 35 cycles at 95°C for 20 seconds, 58°C for 20 seconds, 72°C for 15 seconds, and final extension at 72°C for 10 minutes. The enzyme had to be activated by heat treatment. For this reason, pre-incubation at 95°C for 7 minutes was necessary. In the case of seminested PCR approaches, 0.5 µl of the initial products of the first round of PCR was used as a template in the second round and the PCR programme was comprised to 20 cycles with the same time and temperature conditions as for the first round. The reaction was performed in a T*personal* Combi thermocycler (Whatman® Biometra, Germany).

Post-amplification steps and gel electrophoresis. The PCR products were diluted with loading buffer and appropriate molecular weight marker HyperLadder V (Bioline, USA Inc.) were loaded onto 2 or 3% agarose TBE gel (SERVA Electrophoresis, Germany) containing ethidium bromide (Amresco Inc. Solon, OH, USA) and run at 120 V for 50-60 minutes. The PCR products were then visualized on an ultra-violet light illuminator CCD camera Diana II (Raytest, Straubenhardt, Germany), (Figure 1 and 2).

Table 1. Sequences of Primers

Housekeeping genes	
CF 20i-5'	: 5' -GGT CAG GAT TGA AAG TGT GCA -3'
CF 20i-3'	: 5' -CTA TGA GAA AAC TGC ACT GGA -3'
IgH genes	
JH1-S	: 5' -CTG TCG ACA CGG CCG TGT ATT ACT G -3'
JH2-S (FR-2A)	: 5' -TGG (AG)TC CG(CA) CAG (GC)C(TC) (TC)CN GG -3'
JH -A	: 5' -AAC TGC AGA GGA GAC GGT GAC C -3'
FR 3A	: 5' -ACA CGG C(CT)(GC) 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'
TCR γ genes	
V γ 11	: 5' -TCT GG(AG) GTC TAT TAC TGT GC -3'
J γ 11	: 5' -CAA GTC TTG TTC CAC TGC C -3'
TCR β genes	
Tβ V	: 5' -TGT A(CT)C TCT GTC CCA GCA G -3'
Tβ D1	: 5' -CAA AGC TGT AAC ATT GTG GGG AC -3'
Tβ D2	: 5' -TCA TGG TGT AAC ATT GTG GGG AC -3'
Tβ J1	: 5' -ACA GTG AGC C(GT)G GT(CT) CC -3'
Tβ J2	: 5' -AGC AC(GCT) GTG AGC C(GT)G GTG CC -3'

Results

Results are summarized in Table 2. From a total of 91 formalin-fixed, paraffin-embedded tissues examined by PCR method were 55 cases defined as malignant lymphomas (26

Table 2. Results of IgH and TCR Gene Rearrangement

T-cell lymphoma	IgH+	IgH-	N	Tested	TCR+	TCR-	N	Tested
PTCLU		1		1	12			12
MF		1		1	2		3	5
SPTCL					1			1
ALCL		1		1	1		1	2
LyP		1		1	1	1		2
T-LBL					1			1
TL					3			3
Total		**4**		**4**	**21**	**1**	**4**	**26**
B-cell lymphoma	**IgH+**	**IgH-**	**N**	**Tested**	**TCR+**	**TCR-**	**N**	**Tested**
FL	6		1	7				
DLBCL	6	1	2	9		3	1	4
MALT	8			8				
NMZL	1			1				
SMZL		1		1				
CLL/SLL	3			3				
Total	**24**	**2**	**3**	**29**		**3**	**1**	**4**
Non-neoplastic lesions	**IgH+**	**IgH-**	**N**	**Tested**	**TCR+**	**TCR-**	**N**	**Tested**
Total		**16**	**4**	**20**		**10**	**6**	**16**

PTCLU, peripheral T-cell lymphoma, unspecified; MF, mycosis fungoides; SPTCL, subcutaneous panniculitis-like T-cell lymphoma; ALCL, anaplastic large cell lymphoma; LyP, lymphomatoid papullosis; T-LBL, precursor T-lymphoblastic lymphoma; TL, T lymphoproliferation with no definite diagnosis; FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; MALT, extranodal marginal zone B-cell lymphoma; NMZL, nodal marginal zone B-cell lymphoma; SMZL, splenic marginal zone lymphoma; CLL/SLL, chronic lymphocytic leukaemia / small lymphocytic lymphoma. N, no product.

from T-cells and 29 from B-cells) and 36 as non-neoplastic lesions of a largely inflammatory and reactive type. IgH and TCR analysis was performed on 8 samples at the same time. B-cell lymphomas were according to the WHO classification divided to groups: follicular lymphomas (7), diffuse large B-cell lymphomas (9), extranodal marginal zone B-cell lymphomas (8), nodal marginal zone B-cell lymphoma (1), splenic marginal zone lymphoma (1) and chronic lymphocytic leukaemias/small lymphocytic lymphomas (3). Class T consisted of 7 types: peripheral T-cell lymphomas, unspecified (12), mycosis fungoides (5), subcutaneous panniculitis-like T-cell lymphoma (1), anaplastic large cell lymphomas (2), lymphomatoid papulosis (2), precursor T- lymphoblastic lymphoma (1), T-lymphoproliferations with no definite diagnosis (3).

In class B-cell lymphomas, we detected clonal IgH rearrangement in 24 cases, i.e. in nearly 83% of cases, in 2 cases (7%) clonal rearrangement was not detected and in 3 cases (10%) we did not succeed in extracting the DNA. The clonal TCR rearrangement was found in 21 cases, i.e. in 81% of cases in T-cell lymphomas. In just one case of lymphomatoid papulosis (4%) the rearrangement of TCR was not detected. The isolation of the DNA was not successful in four cases of T-cell lymphomas (15%). In none of the four B-cell lymphomas, in which we simultaneously investigated IgH and TCR, we found clonal TCR rearrangement. Similarly, in none of the four treated cases of T-cell lymphomas we detected any clonal IgH rearrangement.

We further tested all 36 different nodal and extra-nodal non-neoplastic lesions. From this number, we tested 20 cases on IgH and 16 on TCR rearrangement. In no case the clonal rearrangement was detected. The extraction of DNA was not successful in 10 cases which is roughly 28% of total sample number.

Discussion

Over a period of 3 years, we have successively introduced the PCR method of IgH and TCR clonal rearrangement detection into haemato-oncological diagnosis. In the first place we wanted to find the efficiency ratio of the method used on bioptic material (FFPE tissues) routinely processed in the laboratory. The reason for this approach was that the majority of haemato-oncological cases are in the form of paraffin-embedded tissues and also all cases delivered to our department for the "second reading" from other hospitals are in the form of FFPE tissues. There is a reduction in the ability to detect gene rearrangement in lymphomas due to formalin fixation, processing that can cause degradation [6, 25, 33] and other changes to DNA as mentioned above. However, the preliminary results presented in this work are encouraging since there was only a small number of cases in which the DNA was so degraded as to not permit PCR method (10% of B-cell lymphomas, 15% of T-cell lymphomas and 28% of non-neoplastic lesions). We can therefore conclude that in our experience the PCR method can be used safely and that the results are valuable for a final diagnosis made on routinely treated laboratory material.

Except the two cases (diffuse large B cell lymphoma from nasopharynx and splenic marginal zone lymphoma), the proof of the clonal rearrangement IgH coincided with morphological-clinical diagnosis. PCR analysis of IgH (and TCR) genes might be hampered by false-negative results because of im-

proper annealing of the primers to the rearranged gene segments [10, 21]. It is also possible that clonality was not detected by PCR in two cases due to a background of abundant polyclonal reactive cells masking the monoclonal population [36-38]. Detection rate of IgH rearrangement was also closely related to the cell origin of malignant lymphomas. Thus, tumor derived from naive lymphocytes, also designated pre-germinal center (pre-GC) naive B cells, express unmutated VH-region genes [39] and show a high detection rate of clonal IgH gene rearrangement by PCR. The other group of tumors derived from memory B cells generated in the GC and characterized by cells bearing somatically hypermutated VH-region genes (GC and post –GC memory B cells) [21, 39], gives a lower rate of clonality by the PCR method [10, 34]. Efficient IgH PCR analysis can be therefore hampered by primer mismatches originating from random sequence variability in the template DNA due to somatic hypermutation in germinal centers [21, 37].

Aside from only one case of lymphomatoid papulosis (LyP) in the class of T cells lymphoproliferation, proof of clonal rearrangement TCR coincided with morphological-clinical diagnosis. It was found that clonal rearrangement of TCR genes of LyP can be detected in approximately half of the patients [40]. The failure to detect a dominant clone in this case does not have to necessarily reflect the absence of clone. One possible explanation is that cutaneous cell lymphomas may often consist of a large number of reactive polyclonal cells that can reduce the signal intensity of the monoclonal rearrangement of the T cell population and a very small cell clone may therefore be below the threshold for detection [32, 41]. Other genetic events such as deletion of the rearranged TCR or clonal expansion prior to TCR gene rearrangement could also explain the absence of a dominant TCR [42].

In the large and various class of nodal and extra-nodal non-neoplastic lesions of mostly inflammatory and reactive changes, we had no case of clonal IgH and TCR rearrangements. However, nearly one third of all cases were damaged due to tissue fixation which leads to the poor DNA extraction and subsequent unsuccessful PCR analysis [21, 35]. Unfortunately, in just these cases the result of the PCR method was crucial for the final assessment of the diagnosis. For example, we succeeded in differentiating uniquely a number of inflammatory dermatoses of lichenoid and psoriasiform type from early lesions of mycosis fungoides by proper use of the PCR method. Similarly, we were able to differentiate reactive cutaneous lymphoid infiltrations from malignant lymphomas.

In conclusion, we can affirm that even if some difficulties can occur during extraction of first-rate DNA from FFPE tissue, the PCR method is very suitable for routinely elaborate this laboratory material. Its influence on the diagnostics of morphologically unclear cases, in particular, could be crucial.

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References

[1] MEDEIROS LJ, CARR J. Overview of the role of molecular methods in the diagnosis of malignant lymphomas. Arch Pathol Lab Med 1999; 123: 1189–1207.

[2] RAMASAMY I, BRISCO M, MORLEY AA. Improved PCR method for detecting monoclonal immunoglobulin heavy chain rearrangement in B cell neoplasms. J Clin Pathol 1992; 45: 770–775.

[3] TAI YC, PEH SC. Immunoglobulin gene rearrangement of lymphoid malignancies: application of the PCR technique to formalin-fixed, paraffin-embedded tissues. J. Clin. Exp Hematopathol 2003; 43: 11–19.

[4] ELENITOBA-JOHNSON KSJ, BOHLING SD, MITCHELL RS, et al. PCR analysis of the immunoglobulin heavy chain gene in polyclonal processes can yield pseudoclonal bands as an artifact of low B cell number. J Mol Diag 2000; 2: 92–96.

[5] BIELAWSKI K, ZACZEK A, LISOWSKA U, et al. The suitability of DNA extracted from formalin-fixed, paraffin-embedded tissues for double differential polymerase chain reaction analysis. Int J Mol Med 2001; 8: 573–578.

[6] SRINIVASAN M, SEDMAK D, JEWELL S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol 2002; 161: 1961–1971.

[7] VANASSE GJ, CONCANNON P, WILLERFORD DM. Regulated genomic instability and neoplasia in the lymphoid lineage. Blood 1999; 12: 3997–4010.

[8] MESSIER TL, O'NEILL JP, HOU SM, et al. In vivo transposition mediated by V(D)J recombinase in human T lymphocytes. The EMBO Journal 2003; 22: 1381–1388.

[9] BASSING CH, SWAT W, ALT FW. The mechanism and regulation of chromosomal V(D)J recombination. Cell 2002; 109: S45–S55.

[10] Van DONGEN JJM et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: Report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003; 17: 2257–2317.

[11] TRAINOR KJ, BRISCO MJ, STORY CJ, MORLEY AA. Monoclonality in B-lymphoproliferative disorders detected at the DNA level. Blood 1990; 75: 2220–2222.

[12] ANGELIN-DUCLOS C, CALAME K. Evidence that immunoglobulin VH-DJ recombination does not require germ line transcription of the recombining variable gene segment. Mol and Cell Biol 1998; 18: 6253–6264.

[13] INLAY M, XU Y. Epigenetic regulation of antigen receptor rearrangement. Clinical Immunology 2003; 109: 29–36.

[14] TONEGAWA S. Somatic generation of antibody diversity. Nature 1983; 302: 575–581.

[15] SADOFSKY MJ. The RAG proteins in V(D)J recombination: more than just a nuclease. Nucleic Acids Research 2001; 29: 1399–1409.

[16] McCARTHY KP, SLOANE JP, KABAROWSKI JHS, et al. The rapid detection of clonal T-cell proliferations in patients with lymphoid disorders. Am J Pathol 1991; 138: 821–828.

[17] McCARTHY KP, SLOANE JP, KABAROWSKI JHS, et al. A simplified method of detection of clonal rearrangements of the T-cell receptor-γ chain gene. Diagn Mol Pathol 1992; 1: 173–179.

[18] AUBIN J, DAVI F, NGUYEN-SALOMON F, et al. Description of a novel FR1 IgH PCR strategy and its comparison with three other strategies for the detection of clonality in B cell malignancies. Leukemia 1995; 9: 471–479.

[19] BAGG A, BRAZIEL RM, ARBER DA, et al. Immunoglobulin heavy chain gene analysis in lymphomas. J Mol Diagn 2002; 4: 81–89.

[20] ZEMLIN M, HUMMEL M, ANAGNOSTOPOULOS I, STEIN H. Improved polymerase chain reaction detection of clonally rearranged T-cell receptor β chain genes. Diagn Mol Pathol 1998; 7: 138-145.

[21] GURBITY TP, BAGDI E, GROEN NA, et al. Increased sensitivity of B-cell clonality analysis in formalin-fixed and paraffin-embedded B-cell lymphoma samples using an enzyme blend with both 5´→3´ DNA polymerase and 3´→5´exonuclease activity. Virchows Arch 2003; 443: 643–648.

[22] BAGG A. Immunoglobulin and T-cell receptor gene rearrangements: minding your B's and T's in assessing lineage and clonality in neoplastic lymphoproliferative disorders. J Mol Diag 2006; 8: 466–475.

[23] BENHATTAR J, DELACRETAZ F, MARTIN P, et al. Improved polymerase chain reaction detection of clonal T-cell lymphoid neoplasms. Diagn Mol Pathol 1995; 4: 108–112.

[24] LORENZEN J, JUX G, ZHAO-HÖHN M, et al. Detection of T-cell clonality in paraffin-embedded tissues. Diagn Mol Pathol 1994; 3: 93–99.

[25] KUSIC B, DOMINIS M, DZEBRO S, et al. Molecular insight into the diagnosis of lymphoma. Int J Mol Med 2003; 12: 667–671.

[26] TAI YC, PEH SC. Feasibility of T-cell receptor γ (TCR γ) gene rearrangement on formalin-fixed, paraffin embedded tissues by PCR assays. Singapore Med J 2003; 44: 250–255.

[27] MITHA N, McGLENNEN RC. Methods to detect clonal gene rearrangements in lymphomas and leukemias. In: Killeen AA, editor. Methods in molecular medicine. Humana Press Inc., Totowa, NJ 2001: 189–209.

[28] GLEIßNER B, MAURER J, THIEL E. Detection of immunoglobulin heavy chain genes rearrangements in B-cell leukemias, lymphomas, multiple myelomas, monoclonal and polyclonal gammopathies. Leukemia and Lymphoma 2000; 39: 151–155.

[29] NIHAL M, MIKKOLA D, WOOD GS. Detection of clonally restricted immunoglobulin heavy chain gene rearrangements in normal and lesional skin. J Mol Diag 2000; 2: 5–10.

[30] WAN JH, TRAINOR KJ, BRISCO MJ, et al. Monoclonality in B cell lymphoma detected in paraffin wax embedded sections using the polymerase chain reaction. J Clin Pathol 1990; 43: 888–890.

[31] DISS TC, PAN L, PENG H, et al. Sources of DNA for detecting B cell monoclonality using PCR. J Clin Pathol 1994; 47: 493–496.

[32] TRAINOR KJ, BRISCO MJ, WAN JH, et al. Gene Rearrangement in B- and T- Lymphoproliferative Disease Detected by the Polymerase Chain Reaction. Blood 1991; 78: 192–196.

[33] MIETTINEN M, LASOTA J. Polymerase chain reaction based gene rearrangement studies in the diagnosis of follicular lymphoma-performance in formaldehyde-fixed tissue and application in clinical problem cases. Pathol Res Pract 1997; 193: 9–19.

[34] THÉRIAULT C, GALOIN S, VALMARY S, et al. PCR analysis of immunoglobulin heavy chain (IgH) and TCR-γ chain gene rearrangements in the diagnosis of lymphoproliferative disorders: result of a study of 525 cases. Mod Pathol 2000; 13: 1269–1279.

[35] ZHOU X-G, SANDVEJ K, GREGERSEN N, et al. Detection of clonal B cells in microdissected reactive lymphoproliferations: possible diagnostic pitfalls in PCR analysis of immunoglobulin heavy chain gene rearrangement. J Clin Pathol: Mol Pathol 1999; 52: 104–110.

[36] VENKATRAMAN L, CATHERWOOD MA, PATTERSON A, et al. Role of polymerase chain reaction and immunocytochemistry in the cytological assessment of lymphoid proliferations. J Clin Pathol 2006; 59: 1160–1165.

[37] SEGAL GH, JORGENSEN T, SCOTT M, et al. Optimal primer selection for clonality assessment by polymerase chain reaction analysis: II. Follicular lymphomas. Hum Pathol 1994; 25: 1276–1282.

[38] CHILD FJ, WOOLFORD AJ, CALONJE E, et al. Molecular analysis of the immunoglobulin heavy chain gene in the diagnosis of primary cutaneous B cell lymphoma. J Invest Dermatol 2001; 117: 984–989.

[39] KÜPPERS R, KLEIN U, HANSMANN ML, et al. Cellular origin of human B-cell lymphomas. The New England J of Med 1999; 20: 1520–1527.

[40] JAFFE ES, HARRIS NL, STEIN H, VARDIMAN JW, editors. Pathology and genetics of tumours of haematopoietic and lymphoid tissues. WHO classification of tumours. Lyon: IARC Press, 2001.

[41] STEINHOFF M, HUMMEL M, ANAGNOSTOPOULOS I, et al. Single-cell analysis of $CD30^+$ cells in lymphomatoid papulosis demonstrates a common clonal T-cell origin. Blood 2002; 100: 578–584.

[42] CHOTT A, VONDERHEID EC, OLBRICHT S, et al. The same dominant T cell clone is present in multiple regressing skin lesions and associated T cell lymphomas of patients with lymphomatoid papulosis. J Invest Dermatol 1996; 106: 696–700.