

## APPLICATION OF POLYMERASE CHAIN REACTION TO DETECT REARRANGEMENT OF IMMUNOGLOBULIN HEAVY CHAIN GENES IN LYMPHOPROLIFERATIVE DISEASE

Salem H. Khalil, MD, FRCPA, FCAP; Karen Siegrist, MT (ASCP) SI; M. Akhtar, MD, FCAP, FRCPA

As part of our routine work-up in the diagnosis of lymphoproliferative disease, we used a rapid polymerase chain reaction (PCR) assay to amplify the DNA fragments of the framework 3 (FR3) region of the immunoglobulin heavy (IgH) chain genes. The assay does not involve hybridization, nested priming, or sequencing of the amplified PCR product. It was performed on 66 specimens of B-cell lymphoproliferative disease, including acute lymphoblastic leukemia, chronic lymphocytic leukemia, multiple myeloma, hairy cell leukemia and follicular lymphoma. Twenty-six specimens of negative controls, including acute myeloid leukemia, chronic myeloid leukemia in myeloid transformation and idiopathic thrombocytopenic purpura, were also analyzed. The assay was performed with 77% sensitivity and 100% specificity. The standard IgH chain gene rearrangement by Southern blot analysis is reserved for the remaining negative cases if clinically indicated. *Ann Saudi Med* 1997;17(4):395-398.

The diagnosis of lymphoid neoplasms often requires, in addition to morphologic and immunophenotypic criteria, the demonstration of the monoclonality of the lymphoid proliferation.

During B-cell development, the DNA nucleotide sequences of immunoglobulin heavy (IgH) chain genes generated by recombination of germ line gene segments, known as variable ( $V_H$ ), diversity ( $D_H$ ) and joining ( $J_H$ ) elements are the molecular basis for the generation of an enormous repertoire of antibody molecules produced by lymphocytes. The result of the  $V_H$ -N-( $D_H$ )-N- $J_H$  rearrangement event is a relatively short but hypervariable IgH-CDR3 gene sequence characterized by a particular B-cell or B-cell precursor clone.<sup>1-4</sup>

The detection of immunoglobulin rearrangement by Southern blot analysis has allowed demonstration of clonality and lineage.<sup>5-11</sup> However, it has a number of limitations. It requires a substantial amount of fresh or frozen tissue, a large quantity of intact, high molecular weight DNA and radioactively-labeled probes that carry with them the problems of safety and disposal. In addition, the Southern blot method is a time-consuming, technically demanding and very expensive assay within the diagnostic setting.<sup>12-14</sup> In order to overcome many of the disadvantages of Southern blot analysis, we utilized a PCR-amplification-based assay for the assessment of IgH

gene rearrangement as a first-line screening method adjuncted by Southern blot assay whenever suspected false-negative results were encountered. This assay is simpler, faster and less expensive than Southern blot analysis.<sup>13,15,17</sup>

### Materials and Methods

**DNA extraction:** High molecular weight DNA was isolated from fresh peripheral blood and bone marrow aspiration and formalin-fixed paraffin-embedded lymph node tissue samples according to standard proteinase K digestion and organic extraction procedures.<sup>18</sup>

**PCR amplification of immunoglobulin heavy chain gene:** Enzymatic amplification of the IgH gene was performed in a Perkin Elmer GeneAmp PCR System 9600 using a single  $V_H$  primer homologous with a highly conserved sequence near the 3' end of FR3 in conjunction with a single consensus  $J_H$  primer. The sequences were  $V_H$ 5' CTG TCG ACA CGG CCG TGT ATT ACT G 3' and  $J_H$ 5' AAC TGC AGA GGA GAC GGT GAC C 3'.<sup>14</sup> The consensus  $V_H$  and  $J_H$  primers were synthesized by Oligos, Etc. (Portland, Oregon, USA). The reaction mixture contained 0.5  $\mu$ g of DNA; 10mM of Tris-HCl, pH 8.3; 200  $\mu$ M of each dNTP; 50 mM KCl; 1.5 mM  $MgCl_2$ ; 10 pmol/L of each primer and 1 U Taq polymerase (Ampli-Taq; Perkin Elmer-Cetus, Norwalk, CT, USA) in a final volume of 25  $\mu$ L. The reaction mixture was subjected to 30 cycles of PCR amplification following an initial five-minute denaturation step of 94°C. Each cycle consisted of a 30-second denaturation step at 94°C, a 30-second annealing step at 60°C, and a one-minute elongation step at 72°C. The last cycle was followed by a nine-minute elongation step at 72°C. Ten microliters of PCR-amplified product

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From the Department of Pathology and Laboratory Medicine, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia.

Address reprint requests and correspondence to Dr. Khalil: King Faisal Specialist Hospital and Research Centre, MBC 10, P.O. Box 3354, Riyadh, Saudi Arabia.

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was resolved by electrophoresis on a 6% polyacrylamide gel, stained with ethidium bromide and visualized under ultraviolet light. A discrete band within the predicted size range of 90 to 160 base pairs indicated a monoclonal B-cell process.<sup>14</sup> All samples were subjected to amplification using primers for the *erbB-2* gene to confirm that amplifiable DNA was present.

Using DNA isolated from blast cells of a B-cell acute lymphoblastic leukemia, 10%, 5%, and 1% sensitivity controls were prepared by dilution in DNA from the buffy coat of a healthy donor. A monoclonal band was consistently detected in the 10% and 5% sensitivity samples and frequently in the 1% sample, indicating sensitivity of 1% and 5%.

### Results

DNA from 66 specimens with B-cell lymphoproliferative disease confirmed by morphology and immunophenotyping were subjected to PCR amplification using primers for the framework 3 (FR3) sequence of  $V_H$ . The different diagnostic categories and percentage of positivity obtained for IgH gene rearrangement are shown in Table 1. Except for the seven cases of the paraffin-embedded, formalin-fixed follicular lymphomas, all other samples were of fresh material obtained from either peripheral blood or bone marrow aspiration.

Discretely sized amplified products (one or two IgH-VDJ-PCR bands) ranging from 90-160 bp (Figure 1), indicative of a clonal population with rearranged IgH gene, were evident in 51 of the 66 specimens with B-cell lymphoproliferative disease. The PCR assay did not show any discrete band in samples isolated from the 23 acute

myeloid leukemia patients, the two chronic myeloid leukemia in myeloid transformation patients and one ITP patient. Therefore, no false-positive results were detected.

We were able to demonstrate positive signals at a dilution down to 1%-5% when stained with ethidium bromide and visualized under UV light (Figure 2).

### Discussion

The diagnosis of lymphoid neoplasms often requires, in addition to morphologic and immunophenotypic criteria, the demonstration of the monoclonality of the lymphoid proliferation.<sup>5-7</sup> Southern blot analysis of tissue has provided a useful adjunct to diagnosis.<sup>5-11</sup> Although this technique has been very powerful and successful, it has several drawbacks, including the requirement of a large amount of intact high molecular weight DNA, and highly skilled laboratory staff. It is also very expensive and carries with it the problems of safety and disposal of the radioactive materials. The turnaround time of this technique is approximately 7-14 days, a period not suitable for the diagnostic molecular setting.<sup>13-17</sup> The development of a reliable PCR technique for the routine detection of clonal immunoglobulin heavy chain and T-cell receptor chain gene rearrangements represented an attractive alternative to Southern hybridization analysis, because of the relative simplicity of PCR protocols, the short turnaround time (approximately 1-3 days) and the cost-effectiveness of the procedure.<sup>14,16,19,24</sup> However, the main drawback to the use of the PCR technique has been the high rate of false-negative results. Because of the large number of different  $V_H$  and, to a much lesser extent,  $J_H$  segments, it is virtually impossible to use specific primers for PCR amplification of the rearranged IgH locus. There are four relatively conserved regions, called frameworks (FR1 to FR4), between the hypervariable regions (CDR1 to CDR3). By using primers directed against these FR regions, it is possible to amplify V-D-J rearrangements and detect a monoclonal proliferation by a simple DNA-based PCR reaction. One of the most widely used strategies is amplification of the CDR3 region with FR3 and  $J_H$  consensus primers.<sup>25,26</sup> Several studies have evaluated the use of different polymerase chain reaction methods for their efficiency in detecting monoclonality in a well-characterized panel of frozen and paraffin-embedded B-cell lymphoid proliferation.<sup>27-30</sup> These studies included evaluation of primers for the FR1, FR1f, FR1c, FR2, FR3, FR3A, and FR256. Other approaches have used the combination of PCR and temperature gradient gel electrophoresis (TGGE) for the identification of monoclonal PCR products.<sup>31</sup> Using a single pair of primers to the FR3 region and the  $J_H$ , we were able to detect 51 of 66 (77%) morphologically and immunophenotypically proven B-cell lymphoproliferative disease. This compares

TABLE 1. Positivity obtained for IgH gene rearrangement.

Diagnosis	Number of specimens	Percentage
New B-ALL	32/38	84
Relapsed B-ALL	6/9	67
B-CLL	5/5	100
Multiple myeloma	3/5	60
HCL	2/2	100
Bcl-2-positive FL	3/7	43
AML	0/23	0
CML/myeloid transformation	0/2	0
ITP	0/1	0
Total	51/66	77

ALL=acute lymphoblastic leukemia; CLL=chronic lymphocytic leukemia; HCL=hairy cell leukemia; FL=follicular lymphoma; AML=acute myeloid leukemia; CML=chronic myeloid leukemia; ITP=idiopathic thrombocytopenic purpura.

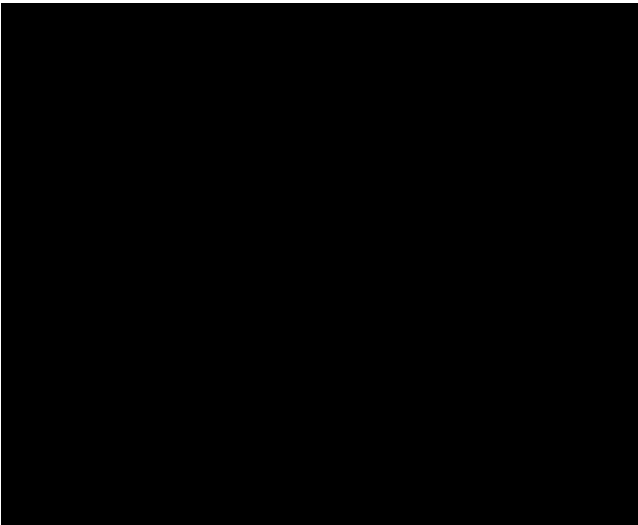


FIGURE 1. Ethidium bromide-stained 6% polyacrylamide gel of PCR products amplified with the IgH primers  $V_H$  and  $J_H$ . Lanes 1) 100 base pair ladder; 2) myeloproliferative disorder; 3) chronic lymphocytic leukemia; 4) pre-B-cell acute lymphoblastic leukemia in relapse; 5) common acute lymphoblastic leukemia in relapse; 6) hairy cell leukemia; 7) follicular lymphoma; and 8) water. Discrete band between 90-160 bp indicates presence of a monoclonal population. Lanes 3,4,5,6 and 7 demonstrate discrete band, while lanes 2 and 8 do not show any band.

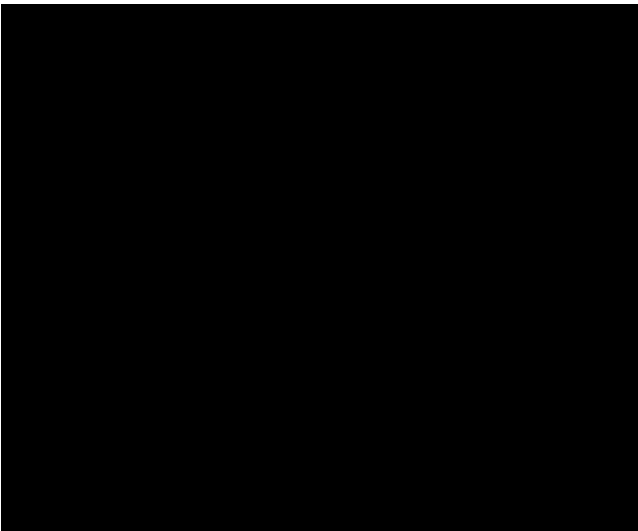


FIGURE 2. Ethidium bromide-stained 6% polyacrylamide gel of PCR products amplified with the IgH primers  $V_H$  and  $J_H$ . Lanes 1) 100 base pair ladder; 2) B-cell acute lymphoblastic leukemia; 3) positive control 5% sensitivity; 4) positive control 1% sensitivity; 5) normal DNA; and 6) water.

well with other laboratories using the same or similar primer pairs.<sup>13,14,16,29,32-34</sup>

Non-Hodgkin's lymphomas were more likely to be negative by FR3-PCR (false negatives) than other B-cell lymphoproliferative disease.<sup>35</sup> This has been attributed to a high rate of mutation or deletion basis from the 3' of  $V_H$  during rearrangement of IgH genes in cases of NHL. This would result in less efficient annealing of the FR3 primer

due to base pair mismatching.<sup>17</sup> This high frequency of false negatives is seen more in the low-grade B-cell lymphomas, which could be attributed to chromosomal translocations involving the immunoglobulin heavy chain gene locus on chromosome number 14, such as t(14;18), seen in follicular lymphomas. It has been demonstrated that in about 10% to 30% of the follicular lymphomas with a t(14;18), the  $J_H$  segment rearranged on only one allele by fusion to Bcl-2 gene.<sup>36</sup> Consequently, these cases do not express immunoglobulin and the IgH/CDR3 PCR fails due to the lack of  $V_H$ - $D_H$ - $J_H$  junctions. All seven cases of follicular lymphomas we have studied were of paraffin-embedded lymph node tissues and all have demonstrated Bcl-2 gene rearrangement of the major breakpoint by PCR amplification and hybridized with a specific probe which is detected by chemiluminescence technique. By excluding those seven cases of follicular lymphomas for the above-mentioned reasons, the overall percentage of positivity is raised to 81%, comparable to the study reported by Sioutos et al.<sup>14</sup>

Somatic mutation is observed in multiple myeloma, leading to a lower rate of detection of IgH gene rearrangement, but unlike follicular lymphoma, the majority of clonal B-cells demonstrate the same mutations.<sup>37</sup> The higher detection rate observed in myeloma (60%), as compared to follicular lymphoma (43%), would suggest that both somatic mutation and V-D-J oligoclonality are responsible for the failure to detect follicular lymphoma rearrangements.

Although combined PCR analysis using more than one set of primer pairs from different FR regions increases the rate of detection of clonality up to more than 90% of the cases,<sup>27</sup> a small proportion of B-cell lymphoproliferative diseases will remain falsely negative. This strategy increases the workload of the laboratory staff and delays the turnaround time, as well as increasing the expenses. For the above-mentioned reasons, we decided to use a simple PCR technique with a single pair of primers achieving sensitivity of 70%-80% and a specificity of 100%, leaving the rest of the cases for Southern blot analysis if it remains clinically indicated.

In conclusion, although we believe that PCR methodology will serve as a very rapid screening procedure in a diagnostic molecular laboratory, there is still room for establishing Southern blot hybridization techniques in diagnostic setting as a back-up for false-negative results.

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