

Detection of immunoglobulin gene rearrangement in B-cell malignancies

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Abstract

Rearrangement of the immunoglobulin heavy chain (**IgH**) gene has been used as a marker of lineage and clonality in the diagnosis of B lymphoproliferative disorders. A number of PCR-based techniques have been developed to overcome the disadvantages of Southern blotting, the standard technique in detecting **IgH** gene rearrangement. Using an established seminested PCR technique with consensus primers to the V and J regions of the **IgH** gene, we analysed DNA prepared from peripheral blood and/or bone marrow specimens from 30 cases of known B cell malignancies (16 chronic lymphocytic leukemia, 11 acute lymphoblastic leukemia and 3 Non-Hodgkin Lymphoma), 3 cases of T lymphoproliferative disease and 3 cases of reactive lymphocytosis diagnosed in Hospital UKM to detect rearranged **IgH** gene. We found that monoclonality as represented by the presence of rearranged **IgH** gene were demonstrated in all the 30 cases. The PCR findings showed 100% concordance with the Southern blot analysis results which also showed rearranged **IgH** bands in all the 30 cases. We also found that none of the cases of T lymphoproliferative diseases and reactive lymphocytosis showed presence of rearranged **IgH** band, suggesting that the amplification using the **IgH** primers is lineage-specific. In conclusion, we find the PCR a useful method to detect **IgH** gene rearrangement in peripheral blood and bone marrow specimen. Since the PCR results are comparable to that of the Southern blotting in demonstrating B cell monoclonality and owing to its many advantages we feel that it can replace the Southern blot technique for the diagnosis of B cell malignancies.

Key words : **IgH** gene rearrangement, B cell lymphoproliferative disorders, DNA methods

INTRODUCTION

The diagnosis of malignant lymphoproliferative disorders is usually made using morphological criteria (histologically and or cytologically) and often supplemented by immunophenotyping. However, in 5 to 10% of cases diagnosis may be difficult even with these methods. Study of the presence of immunoglobulin gene rearrangement can provide further useful information on clonal status, lineage assignment and to a minor degree, extent of the disease. Methods of establishing B cell monoclonality by demonstrating the immunoglobulin gene rearrangement have long been established. One of the earliest methods to be introduced was the Southern blot technique where gene rearrangement was detected by using ³²P-labelled immunoglobulin gene specific probe. Although this method was found to be reliable it has a number of disadvantages. The technique is complex, requires a large amount of DNA and is

time-consuming. The time of one to two weeks taken to obtain the final result decreases its practical value. Subsequent to this a number of polymerase chain reaction (PCR) -based techniques using immunoglobulin heavy chain gene-specific primers have been introduced to amplify the immunoglobulin gene. We report here our experience in the detection of immunoglobulin heavy chain gene rearrangement in 30 cases of B cell lymphoproliferative diseases using an established PCR-based method and Southern blot analysis.

MATERIALS AND METHODS

Samples

We studied DNA extracted from peripheral blood and/ or bone marrow of 37 cases comprising 16 patients with chronic lymphocytic leukaemia (CLL), 11 patients with B – acute lymphoblastic leukaemia (ALL), 3 patients with B-cell non-

Hodgkin's lymphoma (B-NHL), 2 patients with T- acute lymphoblastic leukaemia, 1 patient with T-cell non-Hodgkin's lymphoma, 2 patients with reactive lymphocytosis and 2 normal individuals. Bone marrow specimens were used for all cases of B-ALL, T-ALL B and T NHL and peripheral blood samples were used for all cases of CLL, the 3 cases of reactive lymphocytosis and the 2 normal individuals. Specimens were obtained from patients in the UKM adult Haematology Clinic, Paediatric ward and adult ward in Hospital Kuala Lumpur and Hospital UKM. Diagnosis for all cases were based on conventional peripheral blood and bone marrow morphology, cytochemistry, histological examination and immunophenotyping. A diagnosis of B-lymphoproliferative disease derived from flow cytometric analysis was based upon the presence of surface antigen expression of B cell markers e.g. CD10, CD19, CD 20/22 or CD19/CD5. Mononuclear cells were obtained by Ficoll gradient separation of the blood and bone marrow samples using lymphoprep solution (Sigma). DNA was extracted from peripheral blood and bone marrow mononuclear cells using standard technique.

Southern Blotting

Southern blotting was performed as previously described.^{1,2} 10 ug DNA were digested with restriction enzymes Hind III and Bgl II according to manufacturers instructions (Amersham LIFE SCIENCE). The restricted fragments were electrophoresed on 0.8% agarose gel and transferred to nylon membrane (Hybond, Amersham) and subsequently hybridized to ³²P-labelled Ig heavy chain (Ig JH) gene probe.³ Membrane washings were carried out under high stringent conditions and subsequently exposed to autoradiography.

PCR

Semi-nested PCR were performed according to the method previously described^{3,4} using primers for the amplification of the IgH genes as follows: F R 3 A - 5' A C A C G G C (C / T) (G / C) T G T A T T A C T G T 3' for the 3' end of the V region; L J H - 5' T G A G G A G A C G G T G A C C 3' and V L J H - 5' G T G A C C A G G G T (A / G / C / T) C C T T G G C C C C A G 3' for the 3' end of the J region

PCR condition: The PCR was performed using 1 unit Taq polymerase (Amplitaq, Perkin Elmer Cetus) in 25ul reaction that contained 20 pmol

forward primer FRA3, 20 pmol reverse primer (LJH/VLJH), 200 mmol/l DNTPs mix, 1.5 mmol/l MgCl₂ and 100 ng DNA template. The PCR was performed on the DNA thermocycler (GeneAmp PCR Systems 9700) involving a first round amplification of 30 cycles with primers FR3A and LJH, followed by a second round amplification of 20 cycles with primers FR3A and VLJH. The amplification cycle consists of denaturation temperature of 94°C 1 min, annealing temperature of 55°C 1 min and extension at 72°C 1 min. PCR products were electrophoresed on 2% agarose gel and examined under UV light. The expected DNA fragment sizes are between 100 to 120 bp with a distinct discrete band representing monoclonal B cell expansion and a smear pattern representing a polyclonal B cell population.

RESULTS

DNA from peripheral blood and/or bone marrow specimen from 30 cases of confirmed B-cell lymphoproliferative disease were subjected to both seminested PCR using primers to the V_H and J_H region of the Ig heavy chain gene and Southern Blot analysis using ³²P-labelled IgH J gene probe. A total of 15 cases of B-ALL were seen during the period of study but 4 cases had to be excluded from the study since samples were inadequate for DNA analysis. With PCR analysis discrete sized amplified products ranging from 100 to 120 bp indicative of a clonal population with a common rearranged IgH gene were seen in all the 30 cases and no discrete bands were detected in the DNA samples from 2 cases of T-ALL, the one case of T-NHL, the 3 cases with reactive lymphocytosis and 2 normal peripheral blood (Figs. 1 and 2) where, in all these cases PCR analysis showed presence of only smear on the agarose gel (Fig 2). The absence of false positivity showed that the specificity of the PCR assay is 100%. Southern blot analysis also showed monoclonality as evidenced by the presence of rearranged IgH gene band/s that differ in size from the germline band (Fig. 3) in all the 30 cases studied. There was also no false positivity with Southern blot analysis since none of the T-ALL, reactive lymphocytosis and normal cases demonstrated rearranged IgH gene band. Table 1 shows the results of both PCR and Southern blot analysis on the 37 cases examined.

DISCUSSION

In the germline state, the immunoglobulin heavy chain (IgH) gene exists as discontinuous gene

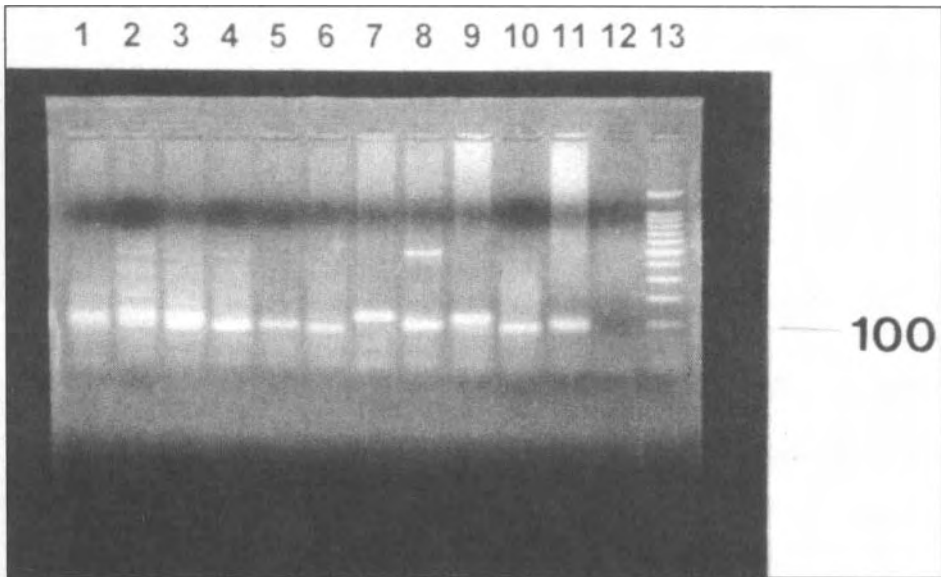


FIG. 1: Gel electrophoresis showing **IgH** gene rearranged bands by PCR amplification. Lane **1** is normal individual and lanes 2,3 are cases of reactive lymphocytosis showing smear pattern. Lanes 4,5,6,7, 8 are cases of B-ALL and lanes 9,10 are cases of B-CLL and showing presence of discrete DNA bands sizes ranging from 100-120 bp. Lane **11**: positive control (known case of B-CLL, **IgH** gene confirmed by DNA sequencing) showing a discrete band of 100 bp. Lane **12**: Negative control (DNA omitted from amplification reaction). Lane **13** contains the 100 bp DNA marker

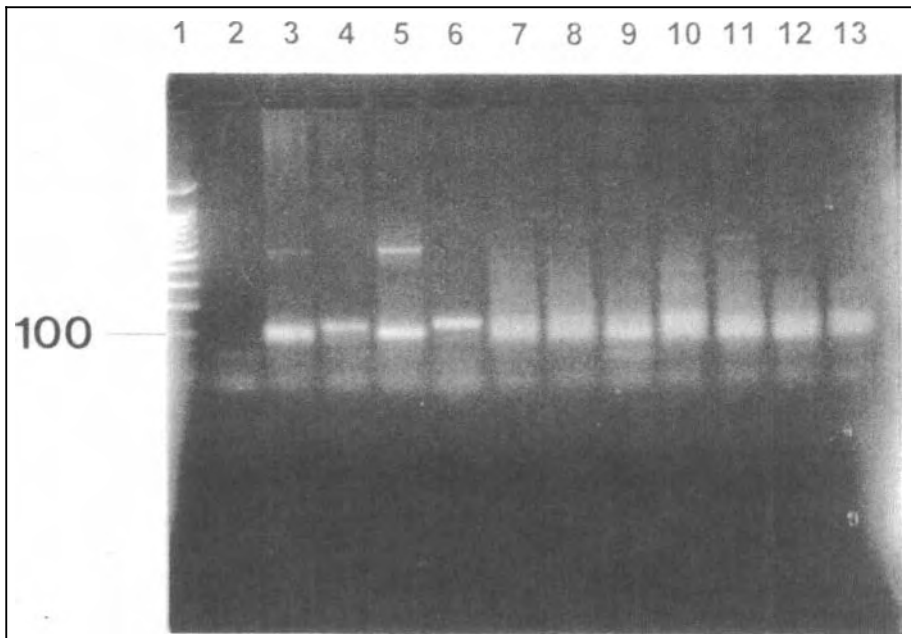


FIG. 2: Gel electrophoresis showing **IgH** rearranged bands by PCR amplification. Lane **1** : contains the 100 bp DNA marker. Lane **2**: negative control (DNA omitted from amplification reaction). Lane **3**: positive control showing discrete band of 100 bp. Lanes **4-6** are cases of B-CLL. Lanes 7,8 are normal individuals; Lanes **9,10** are cases of T-ALL, Lane **11**: case of T-NHL and Lanes **12 & 13** are cases of reactive lymphocytosis, all showing smear pattern.

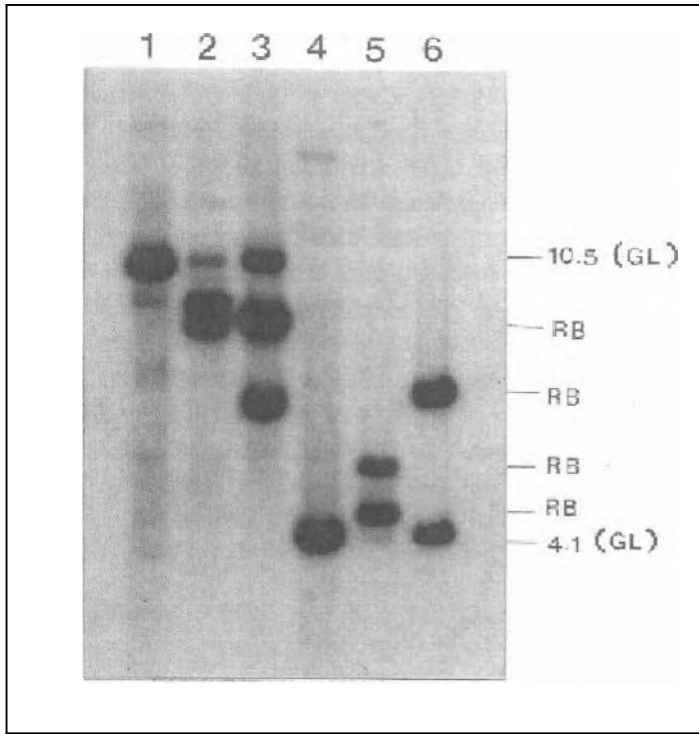


FIG. 3: Autoradiograph of Southern blot analysis for IgH gene rearrangement using restriction enzymes Hind III, Bgl II and probed with ³²P-labelled J_H gene. Lanes 1 –3 contains DNA digested with Hind III and Lanes 4 -6 contains DNA digested with Bgl II. Lanes 1 & 4: DNA from normal individual showing germline bands (GL) of sizes 10.5 kb and 4.1 respectively. Lanes 2 and 5 are from a case of B-CLL, lanes 3 and 6 are from a case of B-ALL: show presence of rearranged IgH gene band (RB).

TABLE 1. Results of IgH gene rearrangement detection by PCR and Southern blotting

Diagnosis	No. Cases	Number of cases showing discrete rearranged IgH gene band			
		Southern blot		PCR	
		Present No. (%)	Absent No.(%)	present	Absent
B-CLL	16	16 (100%)	0	16 (100%)	0
B-ALL	11	11 (100%)	0	11 (100%)	0
B-NHL	3	3 (100%)	0	3 (100%)	0
T-ALL	2	0	2	0	2 (smear)
T-NHL	1	0	1	0	1 (smear)
Reactive	2	0	2	0	2 (smear)
Normal	2	0	2	0	2 (smear)
Total	37	30	7	30	7

NB: Discrete band represents monoclonality with the population of B cells having the same IgH gene rearrangement. A smear results from polyclonal population of B cells having different IgH gene rearrangements.

segments coding for the variable (V_H), **diversity** (D), joining (J_H) and Constant (C_H) regions of the heavy chain **proteins**. However, during B cell differentiation rearrangements occur where one of the many V_H genes combine with a specific D and J_H region to form an active transcriptional heavy chain unit. It was Korsmeyer and co-workers (1981) who first proposed that analysis of pattern of **IgH** gene rearrangement can be used as a powerful and sensitive tool to assess clonality in B cell proliferative **disorders**.^{5,6} The principle of demonstrating monoclonality is based on the fact that all cells in a clonal proliferation have identical **IgH** gene **rearrangements**. In Southern blot analysis using restriction enzymes and ³²P-labelled **IgH** gene probe monoclonality is shown by demonstrating the presence of distinct rearranged DNA bands that differ in size from **IgH** DNA fragments from cells with germ line **configuration**.^{1,7,8} Several groups have recently described methods to detect clonal **IgH** gene rearrangement in B cell malignancies by the PCR gene **amplification**.^{4,9-14} The principles in the study of **IgH** gene rearrangement using PCR are that the forward primer binds to the V region and the reverse primer binds to the J regions of the **IgH** gene, that amplification does not occur unless the gene is arranged such that the V and J segments are in close proximity, that the length of amplified fragments of different gene (hence different B cell) varies depending on the number of nucleotides removed and inserted at the **V(D)J** junctions and that length homogeneity and heterogeneity of the amplified material can be detected by electrophoretic separation, thus enabling distinctions of monoclonality from polyclonality at the gene and cell population level. The excessive number of cells in a single clone produces a discrete band reflecting homogeneity as compared to a normal B cell polyclonal population, where the appearance of a smear corresponds to a heterogeneous population of B cells.

Since its introduction, a number of improvements in the PCR technique have been made to increase the **sensitivity** of detection of clonal B cell proliferation. Such improvement include the use of panels of V_H family specific primers used either sequentially or combined in a single reaction and the use of seminested **PCR**.^{3,4,11,12,15} By performing the seminested PCR method on bone marrow and blood samples as suggested by **Trainor et al**³ using a panel of two V_H specific primers in two rounds of amplification cycles we successfully

demonstrated monoclonality in all the cases of B-cell malignancies (100%) studied. **Trainor et al** in their original report demonstrated monoclonality with the **IgH** primers in only 83% of cases. This difference in rate of detection between our series and that of **Trainor** could be accounted for by the types of tissues studied. In contrast to our study where only blood **and/or** bone marrow specimens were used, **Trainor** examined various tissue samples that included bone marrow, blood and lymph nodes in a total of 52 cases of various B lymphoproliferative disorders. The varied sensitivities of different types of tissues used as the source of DNA was also demonstrated by **Angulo et al** when he observed good sensitivity of the PCR method when blood and bone marrow tissue were used.¹⁰ Apart from tissue type, tumour type is also known to play a part in yielding positive results. This could be a factor contributing to the high rate of positivity seen in our study where the majority of cases were CLL and ALL and only 3 cases were B-NHL. **Diss et al** found that tumour type influenced the sensitivity of PCR detection of clonal **B-cell** population when they studied low and intermediate grade lymphomas." By using a combination set of primers, they found that only 87% cases of **MALT,CB/CC** lymphomas showed rearranged bands. This finding supported the conclusion by **Deanne et al** that lymphoma types are less likely to support amplification than **others**.¹² In contrast, using only **FR3-J_H** primers, **Angulo et al** were able to demonstrate all 20 CLL specimens as clonal whereas the detection rate of small lymphocytic lymphoma and high grade lymphoma were 83% (516) and 60% **respectively**.¹⁰ Similar findings were shown by **Liang et al** when he detected monoclonality by PCR in 15 of 15 cases of CLL and 14 of 17 cases of B-ALL studied.¹⁴

Our findings also suggest that monoclonal amplification with **IgH** primers is **lineage-specific** having observed rearranged bands in 30 of 30 cases of B lymphoproliferative disease and none of the 3 cases of T-lymphoproliferative and 2 cases of reactive lymphocytosis. This appear to support the findings by **Trainor et al** who found that none of his 11 cases of T lymphoproliferative disease showed monoclonal amplification with **IgH** primers. Similarly, **B-lineage** specificity of the **IgH** primers were demonstrated by **Angulo et al** when he performed PCR using the VLJH primers on DNA from 29 reactive cases, 14 normal tissue samples and 8 normal peripheral blood samples and observed

only smears on gel and no discrete rearranged **IgH** gene band.¹⁰

Comparison of the seminested PCR findings with the results of the Southern blot analysis on all the **30** samples showed **100%** concordance. Southern blot analysis performed on the **30** samples showed presence of rearranged **IgH** gene bands that differ from the **germline** bands in **all** the **30** cases. The Southern blot technique is complex, time consuming, requires a large amount of DNA and usually involves the use of **radioisotopes**.⁸ The development of a reliable PCR technique for the routine detection of clonal **IgH** gene rearrangement would represent an attractive alternative to Southern blot analysis. PCR has the advantage of being a simpler technique and rapid to perform providing quick results, requires a small amount of DNA (100 ng), precludes the use of radioactive material and is less **costly**.^{1,8,10} Angulo *et al* carried out a study to compare the costs between the two techniques where, based on tests performed on **3** samples examined simultaneously, he found that for the Southern blot method the reagent and the labour cost were seven and three times more than the cost for PCR **respectively**.¹⁰

From our findings, it appears that a strong case can be made for the seminested PCR to be used as a primary technique in detecting B cell monoclonality. However, it is important to note that from our study such conclusion can be made only for cases using blood **and/or** bone marrow as the source of DNA. Local studies have yet to be carried out to prove if it is true for other materials such as fixed and cytologic specimens. Since there were only **3** cases of **NHL** examined, validity of the finding in **NHL** should be verified with the study of more cases.

In summary, we find the seminested PCR a useful technique for the detection of **IgH** gene rearrangement in blood and bone marrow specimens of B cell malignancies. From our experience we feel that PCR, owing to its advantages should be able to replace the Southern blot technique as a primary technique for the detection of B cell monoclonality.

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