ORIGINAL ARTICLE

Clonality assessment of lymphoproliferative lesions using the polymerase chain reaction: An analysis of two methods

Nikhil Moorchung, R. B. Batra, N. S. Mani, S. S. Gill

Department of Pathology, Armed Forces Medical College, Pune, India.

Address for correspondence:
Lt. Col. Nikhil Moorchung, Department of Pathology, Armed Forces Medical College, Sholapur Road, Pune – 411 040, India.
E-mail: nikhilmoorchung@rediffmail.com

ABSTRACT

Background: Lymphoid malignancies are a heterogeneous group of disorders which may be difficult to differentiate from reactive proliferations even after immunohistochemistry. Polymerase chain reaction (PCR) is believed to be a good adjunct tool for diagnosis. **Materials and Methods:** We examined 24 cases of neoplastic and non-neoplastic lymphoproliferative lesions in this study and evaluated the PCR as an additional tool in the confirmation of the diagnosis. Two different PCR methodologies were evaluated. **Results:** In the evaluation of the T-cell PCR, it was seen that the correlation using both the commercial kits and the custom-synthesized primers was highly significant at a *P* value of <0.05. In the evaluation of the B-cell PCR, it was seen that the correlation using both the commercial kits and the custom-synthesized primers was not significant using either method ($P > 0.05$). **Conclusions:** Both the methods showed an excellent concordance for T-cell γ gene rearrangements, However, the same was not seen in the B-cell receptor rearrangements. This may be because of the small sample size or the inability of consensus V primers to recognize complementary DNA sequences in all of the V segments.

KEY WORDS: Lymphoproliferative lesions, olymerase chain reaction, clonality assessment, polymerase chain reaction

Access this article online
Website: www.ijpmonline.org
PMID: 21623084
DOI: 10.4103/0377-4929.81628
Quick Response Code:

INTRODUCTION

The lymphoid malignancies are a heterogeneous group of disorders that occur as a result of neoplastic transformation of B and T lymphocytes at different stages of B- and T-cell development. The wide variety of lymphoid malignancies reflects the various stages of lymphocyte development and the complexity of the immune system.[1] The ability to diagnose and classify lymphoid malignancies improved substantially in the 1980s because of the development of immunopathological methods utilizing a wide variety of monoclonal antibodies to cell surface antigens.[2] The availability of molecular genetic methods further enhanced our ability to diagnose and classify lymphoid malignancies.[3] The major application of molecular genetic methods in the evaluation of lymphoid neoplasms involves the determination of B- and T-cell clonality.

The B-cell immunoglobulin and T-cell receptors (TCRs) are involved in the process of antigen recognition by normal B and T lymphocytes. These receptors are structurally similar, being heterodimer proteins linked by disulfide bonds, and are composed of both variable (V) and constant (C) regions.[4] The variable regions of these proteins are similarly involved in antigen recognition. The constant region of the immunoglobulin heavy chain protein defines the immunoglobulin classes. The genes that code for the B- and T-cell receptors are also structurally similar and consist of a large number of exons, referred to as a supergene family, that undergo a similar process of DNA recombination leading eventually to the formation of functional receptor proteins.[3-5]

The polymerase chain reaction (PCR) technique is becoming an increasingly popular method for evaluating the presence or absence of B- and T-cell clonality in lymphoid neoplasms.[6,7] This method of DNA analysis allows for the evaluation of minute quantities of DNA by *in vitro* amplification. Short sequences of DNA are shared by nearly all of the V segments that can be recognized by a primer referred to as a consensus V region primer. In a similar fashion, short sequences of DNA shared by nearly all of the J segments can be recognized by a consensus J region primer.[6,7] A polyclonal B- or T-cell population has a large number of rearrangements that differ in size, resulting in a smear pattern. In contrast, monoclonal B- or T-cell populations contain identical rearrangements that result in the formation of a distinct band.

Different PCR based studies reveal considerable variation in many aspects of experimental design and marked differences in the reported results.[8-10] Primarily, single-step, nested and semi-nested techniques are used. Nested or semi-nested techniques allow the detection of template present in small amounts and increase the specificity of the reaction.[11] However, the single-step method is cheap, simple and rapid, and it would be a more desirable assay in screening for cell clonality.

The study was taken up as a pilot study to evaluate the relative usefulness of the PCR as an adjunct in the diagnosis of lymphomas. It was also done to evaluate the relative accuracy of the nested and single-step techniques in confirming the diagnosis of a lymphoma or a non-neoplastic polyclonal cell population. The nested technique was based on a commercially available kit (AB analytica , Via Suizzeia Product code 04-39A and 04-n60 A, Italy) and the single-step method was based on recommended primer sequences.[12] Standardization of the procedures was done in our laboratory.

MATERIALS AND METHODS

Sample Selection

All suspected cases of lymphoma were included in the study. Histopathologic examination had been done in all the samples and this was followed by immunohistochemical evaluation using a panel of, appropriate antibodies when required. The histopathology was the gold standard used for the confirmation of the diagnosis.

DNA Extraction

Fifteen micrometer sections of paraffin-embedded tissue were cut, transferred to a 1.5-mL microcentrifuge tube, and deparaffinized by xylene extraction. Xylene (1.2 mL) was added, and the sample was vortexed and centrifuged at room temperature for 5 minutes to pellet the tissue. Supernatant was removed with a pipette and then the residue was washed twice with 1.2 mL of 100% ethanol to remove xylene. After evaporating the ethanol from the tissue pellet, DNA was extracted using a Qiamp DNA extraction mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. All extracted DNA was stored at −20°C in sterile Tris–ethylenediaminetetraacetic acid (TE) buffer [10 mmol/L Tris and 1 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 8.0].

PCR Amplification

PCR amplification was done using two methods. The first was by using a commercially available kit (AB analytica, Italy) using the nested PCR method. The details of the primers were not provided. One microliter samples of DNA were used per 50 μL PCR reaction. First- and second-round reactions contained 200 μmol/L of each primer, 200 μmol/L dNTP, 2.5 U of *Taq* DNA polymerase, and buffer in a 50 μL reaction. PCR cycling was performed at 95°C for 15 minutes for one cycle, followed by 35 cycles at 95°C for 30 seconds, annealing temperature as stated for 30 seconds, and extension at 72°C for 30 seconds. The final cycle was followed by a 10-minute extension phase at 72°C.

In the second type of PCR amplification, only a single reaction was performed. The details of the primers are as mentioned in Table 1. One hundred nanograms of DNA was used per 50 μL PCR reaction. First- and second-round reactions contained 200 μmol/L of each primer [seven primers for B-cell clonality (JH and Vh 1–6) and five primers for T-cell clonality (JγC and Vγ1–Vγ4)], 200 μmol/L dNTP, 2.5 U of *Taq* DNA polymerase, and buffer (Bangalore Genei , Bangalore) in a 50 μL reaction. PCR cycling was performed at 95°C for 15 minutes for one cycle, followed by 35 cycles at 95°C for 30 seconds, annealing temperature as stated for 30 seconds, and extension at 72°C for 30 seconds. The final cycle was followed by a 10-minute extension phase at 72°C.

Electrophoresis and Imaging

Ten microliter amplification products were visualized under UV illumination after electrophoresis on 3% agarose gel electrophoresis and Tris acetate–ethylenediamine tetraacetic acid (TAE) buffer and staining with ethidium bromide. In order to improve the detection limits, the negative of the images was evaluated.

Statistical Analysis

The SPSS package for Windows (version 13) was used for statistical analysis. Association between the different parameters was evaluated using the Kruskal–Wallis test for nonparametric data. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Twenty-four cases of suspected lymphomas were analyzed. Of the 24 samples, one was an aspirate of a skin lesion, two were fresh skin lesions and the rest were formalin-fixed, paraffin-embedded tissues. The demographic profile of the patients was not available for analysis.

Of the 24 cases, 9 were cases of suspected T-cell lymphomas and 2 were cases of non-neoplastic T-cell proliferations. There were

Table 1: T-cell and B-cell markers

T-cell markers	
JγC	5′ CAA CAA GTG TTG TTC CAC 3′
Vγ1	5′ TGC AGC CAG TCA GAA ATC TTC C 3′
Vγ2	5′ TGC AGG TCA CCT AGA GCA ACC T 3′
Vγ3	5′ AGC AGT TCC AGC TAT CCA TTT CC 3′
Vγ4	5′ TGC AAT TGC ACT TGG GCA GTT G 3′
B-cell markers	
JH	5′ ACC TGA GGA GAC GGT GAC CAG GGT 3′
VH 1	5′ CCT CAG TGA AGG TCT CCT GCA GG 3′
VH 2	5′ GAG TCT GGT CCT GCG CTG GTG AAA 3′
VH 3	5′ GGT CCC TGA GAC TCT CCT GTG CA 3′
VH 4	5′ TTC GGA ACC CTG TCC CTC ACC T 3′
VH 5	5′ AGG TGA AAA AGC CCG GGG AGT CT 3′
VH 6	5′ CCT GTG CCA TCT CCG GGG ACA GTG 3′

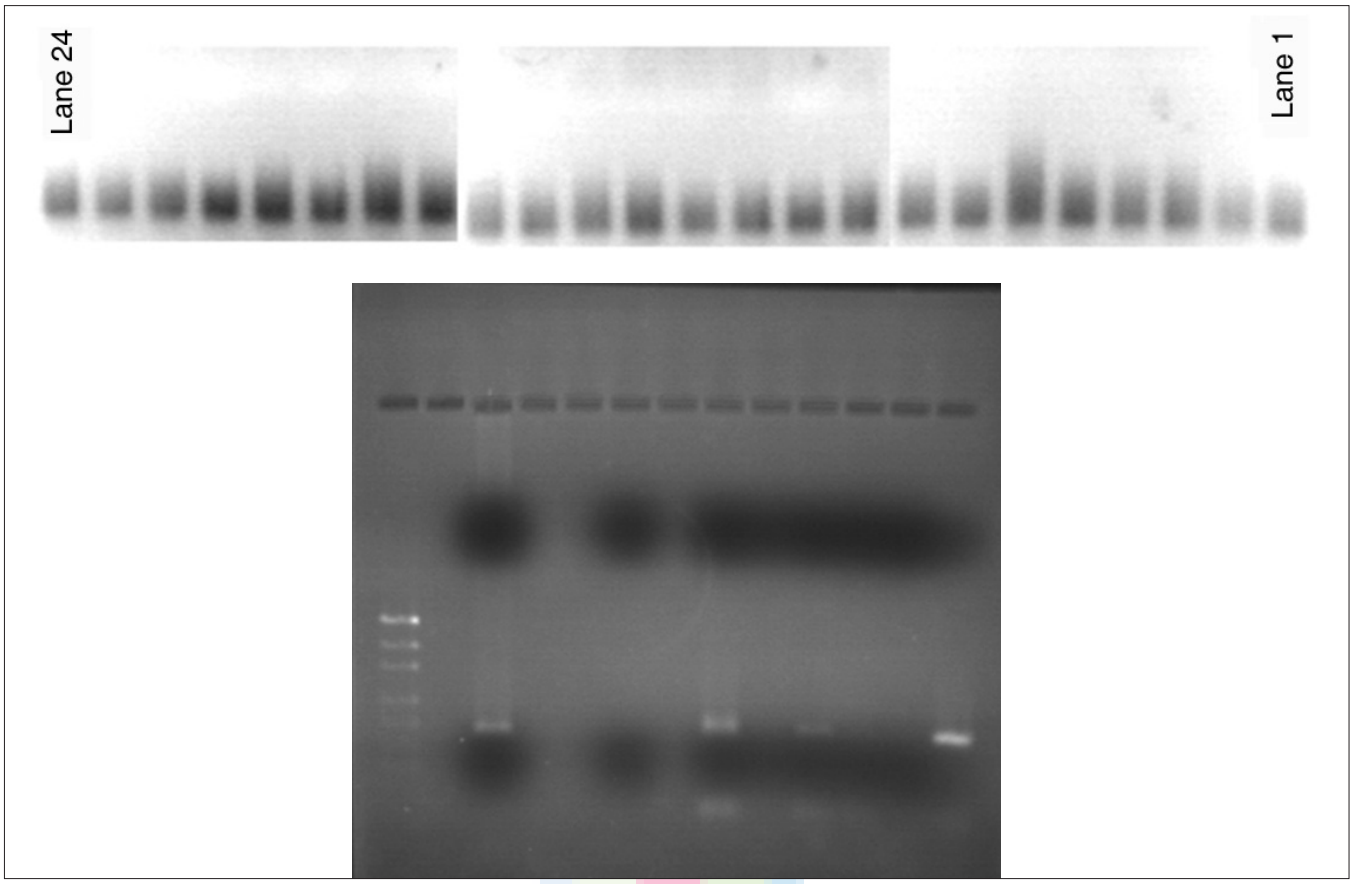

Figure 1: PCR results for clonality testing of T cell run on 3% agarose gel and stained by ethidium bromide. Top – Single step PCR analysis. Lanes 1 to 16 and 22 to 24 show a smear indicating a polyclonal cell population. Lanes 17 to 21 show a dark band along with a smear which was taken as evidence of monoclonality. Bottom – Nested PCR analysis. Lane 1 – DNA ladder. Lanes 3, 8, 10 and 13 show a single clear band indicating a monoclonal cell population

Table 2: A comparison of the results of the kit-based and the custom-synthesized primer PCR

Histopathologic diagnosis	*Positive result by kit*	*Negative result by kit*	*Positive result by custom-synthesized primers*	*Negative result by custom-synthesized primers*
T-cell neoplastic disorders	6	3	5	4
Suspicious for a T-cell neoplastic disorder	2	0	1	1
B-cell neoplastic disorders	6	4	4	6
Suspicious for a B-cell neoplastic disorder	3	0	2	1

10 cases of suspected B-cell lymphoproliferative disorders and 3 cases of non-neoplastic B-cell proliferation.

Of the nine cases of suspected T-cell neoplastic proliferations, six cases showed monoclonality in the commercial kits, whereas five showed a positive result by the custom-synthesized primers [Figure 1 and Table 2]. In the two cases of non-neoplastic T-cell proliferations, both the cases showed a positive result with the commercial kit, but one was negative for a monoclonal band when the PCR was performed using the custom-synthesized primers. The correlation using both the commercial kits and the custom-synthesized primers was highly significant at a *P* value of <0.05.

Of the 10 cases of suspected B-cell neoplastic proliferations, 6 cases showed monoclonality in the commercially available kits and 4 showed a positivity using the custom-synthesized primers. In the three cases of non-neoplastic B-cell proliferations, all the cases showed a negative result with the commercial kit, but two showed a monoclonal band when the PCR was performed using the custom-synthesized primers. The correlation using both the commercial kits and the custom-synthesized primers was not significant (*P* value 0.109 and 0.561, respectively)

The details of the cases and the results are given in Table 3.

DISCUSSION

It has been reported that for the evaluation of B-cell neoplasms,

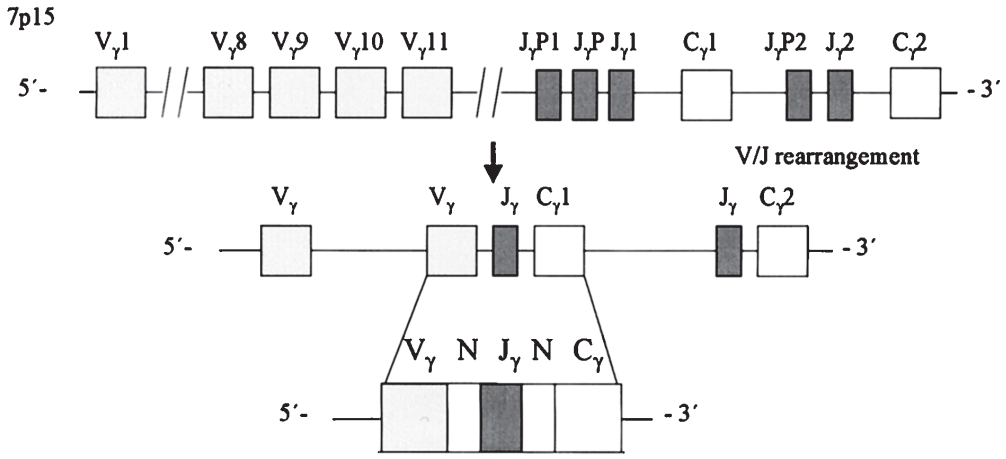

Figure 2: T-cell markers

Table 3: Case details. Table showing the details of all the cases analysed

Type of material	*Diagnosis*	*Commercial kit*		*Custom-synthesized primers*	
		Γ TCR	*CDR III*	*T cell*	*B cell*
FNAC skin lesion	Follicular mucinoses	Negative	Negative	Negative	Negative
Fresh tissue – skin	Follicular mucinoses	Positive	Negative	Negative	Positive
Fresh tissue – skin	Mycosis fungoides (parapsoriasis)	Positive	Negative	Negative	Positive
Paraffin blocks	NHL-T-cell rich B-cell	Positive	Negative	Negative	Positive
Paraffin blocks	Mantle cell lymphoma – Oral cavity	Negative	Negative	Negative	Positive
Paraffin blocks	Atypical lymphoid aggregate – bone marrow	Negative	Negative	Negative	Positive
Paraffin blocks	Soft tissue mass-NHL	Positive	Negative	Negative	Positive
Paraffin blocks	Drug-induced dermatitis	Negative	Negative	Negative	Positive
Paraffin blocks	B-cell lymphoproliferative disorder	Negative	Negative	Negative	Negative
Paraffin blocks	Reactive proliferation	Negative	Negative	Negative	Negative
Paraffin blocks	Gastric Diffuse Large B Cell Lymphoma	Negative	Positive	Negative	Negative
Paraffin blocks	Nasopharyngeal Diffuse large B cell lymphoma	Negative	Positive	Negative	Negative
Paraffin blocks	NHL follicular type Bcl-2 negative	Negative	Positive	Negative	Negative
Paraffin blocks	NHL – diffuse small cell intermediate grade	Negative	Positive	Negative	Negative
Paraffin blocks	NHL – high grade large cell type	Negative	Positive	Negative	Negative
Paraffin blocks	High grade NHL – possibly T cell	Negative	Negative	Negative	Negative
Paraffin blocks	NHL–Diffuse large B cell lymphoma	Negative	Positive	Positive	Positive
Paraffin blocks	NHL–Anaplastic large cell lymphoma	Negative	Negative	Positive	Negative
Paraffin blocks	NHL– Anaplastic large cell lymphoma	Positive	Negative	Positive	Negative
Paraffin blocks	Cutaneous T-cell lymphoma	Positive	Negative	Positive	Positive
Paraffin blocks	Sezary syndrome	Positive	Negative	Positive	Negative
Paraffin blocks	Mycoses fungoides	Negative	Negative	Positive	Negative
Paraffin blocks	Mycoses fungoides follicular variant	Positive	Negative	Negative	Negative
Paraffin blocks	Skin biopsy suggestive of Mycoses fungoides	Positive	Negative	Negative	Negative

two consensus VJ primer sets are used that will detect B-cell clonality in 50–60% of B-cell neoplasms. For the evaluation of T-cell neoplasms, a single multiplex PCR consisting of seven primers specific for V and J segments of the TCR γ gene complex is used. This reaction will detect T-cell clonality in 60–70% of T-cell neoplasms.[13] In the present study, the T-cell evaluation of clonality in both the methods showed similar results. However, evaluation of monoclonality of the B cell did not show results comparable to the published literature. The small sample size may account for this discrepancy. It has also been reported that a high false-negative rate likely occurs because of the inability of consensus V primers to recognize complementary DNA sequences in all of the V segments and the inability of V and J primers to recognize genetic alterations such as partial rearrangements (DJ rearrangements) and chromosomal translocations and somatic mutations involving the antigen receptor gene loci.[14] This could

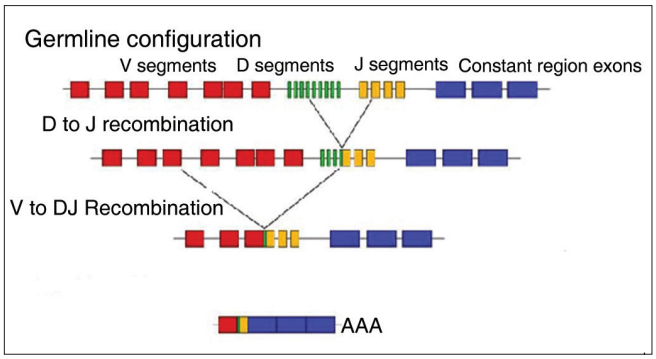

Figure 3: B-cell markers

also account for the results seen in this study. An alternative to PCR in these situations is to do a Southern Blot analysis. However, Southern Blotting is cumbersome and requires large amounts of DNA which may not be available from formalin-fixed, paraffin-embedded tissues.

A problem that we encountered in the present study was visualization of the final product [Figure 1]. Initially, the product was run on a 2% agarose gel; but since it was difficult to observe the monoclonal bands, a 3% agarose gel was used. Using the nested PCR method, it was easy to observe monoclonal bands. However, using the single-step PCR, a dark band was seen along with a smear, indicating a predominant monoclonal population with a surrounding polyclonal cell population.

It has been reported that Polyacrylamide Gel electrophoresis (PAGE) allows higher resolution (i.e. greater discrimination based on product size) and is recommended for smaller PCR products or those with limited size diversity (e.g. TCR γ gene rearrangements).[15] We did not attempt PAGE in this study; however, further evaluation of the single-step PCR will include PAGE as a substitute for agarose gel electrophoresis.

In conclusion, our results using both the methods showed an excellent concordance for T-cell γ gene rearrangements. However, the same was not seen in the B-cell receptor rearrangements for reasons outlined above. Further evaluation using PAGE will evaluate the value of the PCR as an adjunct tool in the diagnosis of lymphomas and leukemias [Figure 2 and 3].

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Source of Support: Nil, **Conflict of Interest:** None declared.