The polymerase chain reaction in the demonstration of monoclonality in T cell lymphomas

T C Diss, M Watts, L X Pan, M Burke, D Linch, P G Isaacson

Abstract

Aims—To evaluate polymerase chain reaction (PCR) amplification of T cell receptor (TCR) β and γ chain genes as a means of demonstrating monoclonality in T cell lymphomas using histological samples; to compare the performance of PCR with Southern blot analysis.

Methods—TCR- β , TCR- γ and immunoglobulin heavy chain (IGH) genes were analysed using PCR in 55 cases of T cell lymphoma (28 frozen tissue and 27 paraffin wax embedded samples), diagnosed using morphological and immunohistochemical criteria. The 28 frozen samples were subjected to Southern blot analysis using TCR- β , TCR- γ and IGH gene probes. Twenty five B cell lymphomas and 21 non-neoplastic lymphoid tissue samples were used as controls.

Results—Using TCR- β PCR, monoclonality was detected in 24 (44%) of 55 T cell lymphomas compared with 43 (78%) of 55 using TCR- γ PCR and in 82% with both techniques. Five (9%) of 55 T cell lymphomas were IGH PCR positive. None of the non-neoplastic lymphoid control samples were PCR positive. All B cell lymphomas showed a polyclonal pattern with TCR- β PCR while a single B cell lymphoma was positive using TCR- γ primers. With TCR- β PCR, a monoclonal result was seen in 12 (43%) of 28 frozen samples of T cell lymphoma, compared with 23 (82%) of 28 using Southern blot analysis. With TCR- γ PCR, 19 (68%) of 28 frozen tissue samples were positive, compared with 26 (93%) of 28 using Southern blot analysis. A single case showed IGH rearrangement by Southern blot analysis. **Conclusion**—TCR- γ PCR should be the method of choice for analysis of clonality in paraffin wax embedded sections of lymphoproliferative lesions, as TCR- β PCR has a high false negative rate. Southern blot analysis remains the most successful technique when sufficient fresh tissue samples and resources are available.

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Distinction between benign and malignant T cell lymphoproliferative disease can be difficult using morphological criteria. For example, low grade T cell lymphomas presenting in the skin can be indistinguishable from re-

active conditions, even with the aid of immunophenotyping.¹ The best available marker of T cell malignancy is T lymphocyte monoclonality, which is rarely encountered in non-neoplastic disease. However, monoclonality of T cells, unlike that of B cells, cannot be reliably deduced by immunophenotyping. Studies of gene rearrangement using Southern blot analysis of restriction fragments of T cell receptor (TCR) α , β , γ , or δ chain genes permit identification of monoclonal populations of T cells.² Unfortunately, these techniques are time consuming, use radioactive isotopes and depend on the availability of fresh/frozen tissue samples, and thus have not progressed from the research laboratory to the diagnostic service. Alternative approaches based on the amplification of TCR genes using the polymerase chain reaction (PCR) have been devised which can also detect monoclonal populations of T cells and are rapid, do not use radioisotopes and can be applied to paraffin wax embedded tissue samples.³⁻⁷ PCR methods are based on size analysis of amplified variable regions and rely on the fact that monoclonal populations give rise to PCR products of discrete size rather than the wide range of product sizes characteristic of polyclonal populations. The TCR- β ,⁵ - γ ^{4,6,7} or - δ ⁸ genes have been the favoured targets for detection of monoclonal T cells as the TCR- α gene is highly complex. For evaluation of T cell lymphomas in general, amplification of TCR- β and - γ is the most promising as the - δ chain gene is frequently deleted in mature T cells.⁹ At present, it is unclear whether targeting the TCR- β or the - γ chain gene offers the most efficient means of demonstrating monoclonality in lymphoproliferative lesions and whether PCR offers a viable alternative to Southern blot analysis. In order to help the pathologist to select the most appropriate techniques, we have extended and evaluated previously published PCR protocols for amplification of TCR- β and - γ chain genes. In cases where frozen, unfixed tissue samples were available, Southern blot analysis has been carried out, using probes to the TCR- β and - γ chain genes, in order to compare PCR with the gold standard technique.

Detection of clonal antigen receptor gene rearrangement can also be used to determine lineage of lymphoproliferation.¹⁰ Some lymphomas, however, carry apparently inappropriate antigen receptor gene rearrangements,¹¹ prompting us to assess PCR amplification of the immunoglobulin heavy chain (IGH) gene in addition to TCR genes.

Department of
Histopathology,
University College
London Medical
School, University
Street, London WC1E
6JJ

T C Diss
L X Pan
P G Isaacson

Department of
Haematology
M Watts
D Linch

Department of
Histopathology, Mount
Vernon Hospital,
Middlesex
M Burke

Correspondence to:
Professor P G Isaacson.

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Methods

Tissue samples were obtained from the archives of the Histopathology Department at University College London Medical School and from a Leukaemia Research Fund study financed by the British National Lymphoma Investigation (BNLI) based at Mount Vernon Hospital, London. DNA extracts from frozen, unfixed tissue samples were made using a standard technique and from paraffin wax blocks using the method of Wright and Manos.¹² DNA samples from 55 cases of T cell lymphoma were analysed, diagnosed using morphological and immunophenotypic criteria and classified according to the revised European-American Lymphoma Classification.¹³ The 28 frozen tissue samples comprised 11 cases of intestinal T cell lymphoma, 12 of peripheral T cell lymphoma, two precursor T lymphoblastic lymphomas, one anaplastic large cell lymphoma, and two cases of mycosis fungoides. The 27 paraffin wax embedded tissue samples included nine peripheral T cell lymphomas, four precursor T lymphoblastic lymphomas, eight anaplastic large cell lymphomas, one case of mycosis fungoides, four angio-immunoblastic T cell lymphomas, and one unclassified T cell lymphoma (necrotic). Twenty five frozen tissue samples of B cell lymphoma with PCR detectable IGH gene rearrangements (five small lymphocytic (B-CLL), five MALT type, five mantle cell, five follicular centre, and five large cell), the T cell lines Jurkatt and CEM-CCRF, and the Raji B cell line were also studied. Twenty one non-neoplastic controls comprised 10 paraffin wax sections of hyperplastic tonsil and 11 peripheral blood lymphocyte samples (seven from healthy donors, three from subjects with infectious mononucleosis and one from a patient with a postsplenectomy lymphocytosis).

POLYMERASE CHAIN REACTION

TCR- β chain gene

Amplification of the TCR- β chain gene was carried out using the technique described by McCarthy *et al*⁵ in five separate reactions with the following primer combinations: V+J1, V+J2, D1+J1, D1+J2, D2+J2 (table 1). Reaction mixtures contained 10 mM Tris/HCl (pH 9), 50 mM KCl, 0.1% Triton-X 100, 250 ng of each primer, 200 μ M of each dNTP, 1–1.5 mM MgCl₂, 100 ng DNA (or 2 and 5 μ l of paraffin wax section extract), and 1 unit of

Taq polymerase (Promega, Southampton, UK) in a total volume of 50 μ l. After an initial denaturation step at 95°C for seven minutes ("hot-start"), the Taq enzyme was added at 58°C followed by 40 cycles of one minute at 93°C, one minute at 58°C, and two minutes at 72°C (seven minutes on the last cycle) on a thermal cycler (Hybaid, Teddington, UK). Products were run on 10% polyacrylamide gels, stained with ethidium bromide and viewed under ultraviolet light. All samples were studied in duplicate.

Samples giving rise to a total of one or two clearly visible, discrete fragments within the expected size range (55 to 100 base pairs) and of the same size in both amplifications were scored as positive (monoclonal); those giving rise to a smear of products or an inconsistent, weak "ladder" pattern of amplification were scored as negative (polyclonal). If no products were seen, the reactions were presumed to have failed and were repeated.

TCR- γ chain

The TCR- γ chain gene was amplified using the method described by McCarthy *et al*⁶ ("PCR γ 1"; table 1, fig 1) using two reactions with primers V γ I+V γ III/IV+J γ 1/2 (product sizes approximately 70–95 base pairs) and V γ I+V γ III/IV+JP γ 1/2 (product sizes 80–110 base pairs). As these primers do not target rearrangements involving the V γ II or JP segments, extra primers were used to investigate their usefulness ("PCR γ 2"; table 1, fig 1). Reactions were carried out as follows: V γ II+J γ 1/2, V γ II+JP γ 1/2, V γ I+V γ III/IV+JP γ (product sizes approximately 150–180, 160–190 and 70–95 base pairs, respectively). After denaturation at 95°C for seven minutes, Taq polymerase was added at 55°C followed by 40 cycles of one minute at 93°C, one minute at 55°C, and one minute at 73°C (seven minutes on the last cycle). Otherwise, reaction mixtures, analysis of products and interpretation were carried out as for the TCR- β analysis with the exception that a MgCl₂ concentration of 1.5 mM was used in all reaction mixtures.

Immunoglobulin heavy chain gene

Amplification of the IGH gene from the framework 3 part of the V segment to the J region was carried out as previously described.¹⁶ Analysis of products and interpretation of results were as described above for TCR- β amplification.

Southern blot analysis

High molecular weight DNA extracts from the 28 frozen samples of T cell lymphoma were digested with restriction enzymes *Eco*RI, *Hind*III and *Bam*HI. Digests were separated on 0.8% agarose gels and blotted onto nylon membranes. Blots were hybridised sequentially to ³²P labelled probe C β for TCR- β ,¹⁷ pH60 for TCR- γ ¹⁸ and J μ for the IGH gene,¹⁹ washed under stringent conditions and exposed to x ray film at –70°C for three to five days. Membranes were stripped of radiolabel by washing in

Table 1 Sequences and sources of primers

Primer	Sequence	Reference
TcR- β		
V	5'-TGTATCTCTGTGCCAGCAG-3'	5
D1	5'-CAAAGCTGTAACATTGTGGGGAC-3'	5
D2	5'-TCATGGTGTAACTTGTGGGGAC-3'	5
J1	5'-ACAGTGAGCCGGTTCC-3'	5
J2	5'-AGCACCGTGAGCCTGGTGCC-3'	5
TcR- γ		
V γ I	5'-TCTGGG/AGTCTATTACTGTGC-3'	6 (V γ 11)
V γ III/IV	5'-CTCACACTCC/TCACTTC-3'	6 (V γ 101)
J γ 1/2	5'-CAAGTGTGTTCCTCCAGTCC-3'	6 (J γ 12)
JP γ 1/2	5'-GTTACTATGAGCT/CTAGTC-3'	6 (JP γ 12)
V γ II	5'-GAAAGGAATCTGGCATTCCG-3'	Short M, personal communication
JP γ	5'-TTGTTCCGGGACCAAATACC-3'	14

Figure 1 (A) Organisation of the germline TCR-γ chain gene. The V and J segments targeted by PCRγ1 primers are shown as open boxes. VγI primer binds to all VγI family members; the VγIII/IV primer binds to V10 and V11 (families III and IV); Jγ1/2 is complementary to Jγ1 and Jγ2; Jγ1/2 binds to JP1 and JP2. Additional segments targeted by PCRγ2 primers are shown as solid boxes; VγII binds to V9 and the JP primer binds to JP. Pseudogenes, VA and VB, are shown as shaded boxes (not targeted) and the constant region clusters (C1 and C2) are shown as large shaded boxes.¹⁵ (B) Rearranged TCR-γ chain gene showing the primer positions which permit amplification of V-N-J regions.

Table 2 Southern blot and PCR results, shown as numbers of monoclonal cases

	n	TCR-β		TCR-γ			IGH	
		Blot	PCR	Blot	PCR1	PCR2	Blot	PCR
T cell lymphoma								
frozen tissue	28	23 (82%)	12 (43%)	26 (93%)	19 (68%)	3 (11%)	1 (4%)	2 (7%)
paraffin wax sections	27	NA	12 (44%)	NA	23 (85%)	1 (4%)	NA	3 (11%)
total	55	—	24 (44%)	—	42 (76%)	4 (7%)	—	5 (9%)
B cell lymphoma	25	—	0 (0%)	—	1 (4%)	0 (0%)	25/25*	25/25*
Controls	17	—	0 (0%)	—	0 (0%)	0 (0%)	—	0 (0%)

* These cases were selected on the basis of a rearranged Ig heavy chain gene.

boiling 0.5% sodium dodecyl sulphate between hybridisations. Following hybridisation to Cβ and JH probes, cases which showed one or two non-germline bands on the radiographs with

one or more restriction digests were scored as positive (monoclonally rearranged) and those showing only germline bands were scored as negative. Because of the limited diversity of the TCR-γ chain gene, polyclonally rearranged T cell populations give rise to a predictable ladder of bands in addition to germline fragments²⁰ which are clearly seen following digestion with EcoRI or HindIII and hybridisation with the pH60 probe. Samples which showed the presence of one or two bands distinctly stronger than the other rearranged fragments with one or more restriction digests were scored as positive and those which showed only a germline or typical polyclonal ladder were recorded as negative.

Results

TCR-β

After optimisation, which required meticulous control of reaction conditions, 24 (44%) of 55 cases of T cell lymphoma analysed using TCR-β PCR showed a monoclonal pattern of one or two discrete bands and the remainder showed a polyclonal pattern of a smear of PCR products (table 2; fig 2). Of the frozen tissue samples, 12 (43%) of 28 were positive compared with 82% positive by Southern blot analysis; the five cases negative on Southern blot analysis were also PCR negative. Twelve (44%) of the 27 paraffin wax samples were PCR positive. In 12 of the 24 samples found to be rearranged on

Figure 2 Polyacrylamide gel of TCR-β PCR products (D2+J2 primers). Lane M, PhiX-HinfI molecular weight markers (100 base pair fragment is arrowed); lane 1, negative control (no DNA); lane 2, hyperplastic tonsil; lane 3, B cell lymphoma; lanes 4 and 5, intestinal T cell lymphomas; lane 6, peripheral T cell lymphoma; and lane 7, angioimmunoblastic T cell lymphoma. Lanes 4 and 5 were interpreted as positive (monoclonal), and lanes 6 and 7 as negative (polyclonal). The weak band visible in lane 7 was not seen on duplication of the reaction and therefore did not satisfy the adopted criteria for monoclonality.

Southern blot analysis, clonal bands were seen with only one of three restriction enzymes. In the fresh tissue extracts primer pairs V+J1,

(A)

(B)

Figure 3 (A) Polyacrylamide gel of TCR- γ PCR products (PCR γ 1 V γ I-III/IV-J1/2 primers). Lane M, *Phi*X-HinI molecular weight markers (100 base pair fragment is arrowed); lane 1, negative control (no DNA); lane 2, hyperplastic tonsil; lane 3, B cell lymphoma; lane 4, intestinal T cell lymphoma; lane 5, mycosis fungoides; lane 6, peripheral T cell lymphoma; and lane 7, angioimmunoblastic T cell lymphoma. Lanes 4, 5 and 6 were interpreted as positive (monoclonal), and lane 7 as negative (polyclonal). (B) Polyacrylamide gel of TCR- γ PCR products (PCR γ 1 V γ I-III/IV-JP1/2 primers). Lane M, *Phi*X-HinI molecular weight markers (100 base pair fragment is arrowed); lane 1, negative control (no DNA); lane 2, hyperplastic tonsil; lane 3, B cell lymphoma; lanes 4 and 5, intestinal T cell lymphomas; lane 6, peripheral T cell lymphoma; and lane 7, angioimmunoblastic T cell lymphoma. Lanes 4, 5 and 6 were interpreted as positive (monoclonal), and lane 7 as negative (polyclonal).

Table 3 Total numbers of cases and alleles amplified by each primer set

TCR gene	Primer set	Cases	Alleles
β	V-J1	1	1
β	V-J2	8	8
β	D1-J1	3	3
β	D1-J2	4	4
β	D2-J2	18	18
γ (PCR γ 1)	V γ I-V γ III/IV-J1/2	25	34
γ (PCR γ 1)	V γ I-V γ III/IV-JP1/2	23	26
γ (PCR γ 2)	V γ II-J1/2	3	3
γ (PCR γ 2)	V γ II-JP1/2	1	1
γ (PCR γ 2)	V γ I-V γ III/IV-JP	1	1
IGH	FR3-JH	5	5

V+J2, D1+J1, D1+J2, and D2+J2 all produced at least one positive result, with D2+J2 being the most useful set (18 of 24 positive results; table 3). In four cases a total of three bands was seen, but in each case an identically sized fragment was amplified with D1+J2 and D2+J2, suggesting that the two D primers sometimes cross-react. All cases which showed a germline Southern blot were PCR negative. The Jurkatt and CEM-CCRF T cell lines were both positive, whereas the Raji B cell line was negative. Using PCR, 25 B cell lymphomas and 21 non-neoplastic lymphoid tissue extracts all showed a polyclonal pattern.

TCR- γ

TCR- γ PCR was easier to optimise and was more robust than TCR- β PCR. Using the method described by McCarthy *et al.*,⁶ 42 (76%) of 55 cases of T cell lymphoma were monoclonal (table 2; fig 3). Occasional cases gave rise to variable ladders rather than product smears. Of the 28 frozen tissue samples, 19 (68%) were monoclonal by PCR compared with 93% by Southern blot analysis. In nine of 26 Southern blot analysis positive cases a rearranged band was seen in only one restriction enzyme digest. Both samples negative on Southern blot analysis were also PCR negative. Twenty three (85%) of 27 paraffin wax samples were PCR positive. Supplementing the PCR analysis of TCR- γ with primers V γ II and JP γ (PCR γ 2) increased the overall detection rate of TCR- γ PCR from 76 to 78%. Although five cases showed a monoclonal pattern using these primers (table 3), only one was negative with PCR γ 1. Both T cell lines were positive and the B cell line negative. One of the 25 B cell lymphomas (a large cell tumour), though none of the 21 non-neoplastic lymphoid samples, showed a dominant band. Use of both TCR- β and - γ PCRs together permitted the detection of 82% of T cell lymphomas.

IMMUNOGLOBULIN HEAVY CHAIN GENE

Five (9%) of 55 T cell lymphomas showed clonal rearrangement of the IGH gene, as determined by PCR; the remainder produced a smear of products. The monoclonal cases comprised one intestinal, two precursor and two angioimmunoblastic T cell lymphomas. All genotypic data for these cases are shown in table 4. Of 28 frozen tissue cases analysed, two were PCR positive, one of which showed J_H rearrangement on Southern blot analysis.

PCR results for each disease group are shown in table 5.

Discussion

Previous PCR based investigations have shown monoclonality in 50–76% of T cell lymphomas using TCR- β primers^{5,21} and in 58–100% using TCR- γ primers.^{4,6,7,21–23} However, these studies have assessed few cases, or have analysed a single disease entity (mycosis fungoides²³), a single TCR gene^{4,5,7,22} or have presented no Southern blot data.²¹ The present study is the

Table 4 Results of PCR and Southern blot analysis of immunoglobulin (Ig) heavy chain positive T cell lymphomas

Case	Diagnosis	β -blot	β -PCR	γ -blot	γ -PCR	Ig-blot	Ig-PCR
1	Intestinal T cell lymphoma	R	—	G	—	G	+
2	Precursor T lymphoblastic lymphoma	R	—	R	—	R	+
3	Precursor T lymphoblastic lymphoma	ND	—	ND	—	ND	+
4	Angioimmunoblastic T cell lymphoma	ND	—	ND	+	ND	+
5	Angioimmunoblastic T cell lymphoma	ND	—	ND	+	ND	+

R=rearranged; G=germline; ND=not done (no fresh tissue available).

first to provide a direct comparison between detection of TCR- β and TCR- γ rearrangements using both PCR and Southern blot analysis in a range of lymphoma types.

On PCR analysis of 55 cases of T cell lymphoma monoclonality was detected in 44% of cases with TCR- β and in 78% with TCR- γ primers. Thus, TCR- β PCR was significantly less sensitive in demonstrating monoclonality than TCR- γ PCR. This may be because the TCR- β primers are consensus sequences targeted to a large number of diverse germline segments,⁵ whereas the TCR- γ primers fit closely to a limited number of targets.⁶ It is also possible that a higher proportion of malignant T cell clones carry TCR- γ chain gene rearrangements than β , a view supported by Theodorou *et al.*²⁴ A further disadvantage of TCR- β PCR is the requirement for five primer pairs in the analysis, although a single set (D2+J2) detected 75% of the TCR- β positive cases.

Both TCR- β and TCR- γ PCR results using paraffin wax embedded tissue samples were comparable to those obtained using frozen, unfixed tissue, confirming that these techniques are suitable for routinely processed specimens.^{21,22}

Monoclonal bands were not seen in B cell lymphomas using TCR- β PCR. This suggests that the technique can help to define lymphocyte lineage in histological material, although the high incidence of false negatives will reduce its usefulness. A single case of B cell lymphoma showed clonal rearrangement of the TCR- γ chain gene. Previous studies have shown that a proportion of B lineage tumours carry rearranged TCR- γ chain genes, notably in B lymphoblastic disease.²⁵

No false positive TCR- β or TCR- γ PCR results were seen in 21 non-neoplastic lymphoid tissue samples, although we have recorded an unexpected reproducible dominant band using TCR- γ PCR in a case of hepatitis, with no evidence of involvement by lymphoma (unpublished data). Furthermore, analysis of intestinal intraepithelial lymphocytes from patients with non-lymphomatous, non-inflammatory disease has shown that dominant

clones are present within T cell subsets, presumably responding to restricted numbers of antigens.²⁶ Studies to determine whether such clones may be detectable in small gastrointestinal biopsy specimens using the methodology described here are in progress. Monoclonal populations of T cells have also been described in peripheral blood specimens of elderly subjects.²⁷ Although results to date suggest that PCR detectable monoclonality is rare in non-neoplastic T cell disease, further studies are necessary to clarify this issue. It is evident that the presence of a dominant T cell clone does not necessarily equate to malignancy, therefore close correlation of PCR analysis with standard histological assessment is essential to minimise the risk of misdiagnosis.

The addition of extra primers to the TCR- γ analysis (PCR γ 2) did not significantly increase the detection rate (from 76 to 78%). Further refinement of PCR techniques for amplification and analysis of TCR- γ chain genes may permit detection of a higher proportion of rearrangements. For example, by mixing multiple primers targeting all predictable rearrangements in single tube reactions or by the use of GC-clamped primers designed to enhance separation on denaturing gradient gels.²⁸ However, improvements in sensitivity by alternative methods of product analysis may more frequently lead to detection of expanded clones which do not correspond to neoplasia. Such methods may therefore be more appropriate during follow up rather than at diagnosis. It may be necessary to include quantitative criteria in the definition of dominant clones, rather than relying on the sensitivity of the methodology to provide the threshold.

Our finding of IGH gene rearrangement in 9% of our T cell lymphomas is perhaps surprising. Misdiagnoses on morphology and immunophenotype are a possibility, although, in all but one case, TCR gene rearrangement was also shown by PCR or Southern blot analysis or both (table 4). In the light of previous reports in which IGH gene rearrangements have been observed in precursor and angioimmunoblastic T cell lymphomas,^{29,30} it is evident that IGH rearrangements in these tumours are not uncommon. Four of the five IGH rearrangements observed here were in these lymphoma subtypes. A further possibility is that minor dominant B cell clones were present in these samples, although in one of the two cases studied using Southern blot analysis a J_H rearrangement was demonstrated, confirming the presence of a major IGH rearranged clone. These observations reinforce the view that a T or B cell phenotype cannot be inferred from a

Table 5 PCR results for each T cell lymphoma subtype studied

Lymphoma	TCR- β	TCR- γ *	IGH
Intestinal T cell lymphoma	5/11 (45%)	6/11 (55%)	1/11 (9%)
Peripheral T cell lymphoma	8/21 (38%)	18/21 (86%)	0/21 (0%)
Precursor T lymphoblastic lymphoma	2/6 (33%)	4/6 (67%)	2/6 (33%)
Anaplastic large cell lymphoma	4/9 (44%)	7/9 (78%)	0/9 (0%)
Mycosis fungoides	2/3 (67%)	3/3 (100%)	0/3 (0%)
Angioimmunoblastic T cell lymphoma	2/4 (50%)	4/4 (100%)	2/4 (50%)
Unclassified T cell lymphoma	1/1 (100%)	1/1 (100%)	0/1 (0%)
Total	24/55 (44%)†	43/55 (78%)†	5/55 (9%)

* PCR γ 1 + PCR γ 2; †PCR- β and PCR- γ together 45/55 (82%).

single antigen receptor amplification, although the presence of a TCR or IGH rearrangement in the absence of the other may be regarded as strong evidence of, respectively, a T or B cell lineage.

Southern blot analysis was more successful at detecting TCR- β or - γ rearrangements than PCR, presumably because a lack of primer binding caused false negative PCR reactions in a proportion of cases. However, as the Southern blot technique may present problems of interpretation³¹ and cannot be applied to paraffin wax embedded material, the PCR approach is of more practical use in histopathology.

In conclusion, TCR- γ PCR should be the method of choice for analysis of clonality of T cell lymphoproliferations in paraffin wax embedded tissue samples, as it permits amplification of a higher proportion of malignant clones than TCR- β PCR. A simple approach using two primer sets is sufficient to detect most rearrangements, although extra primers may be of use in a minority of cases. Southern blot analysis remains the most successful technique when fresh/frozen tissue samples and resources are available.

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