Resolving T-cell receptor clonality in two and genotype in four multiplex polymerase chain reactions

Michael Dictor
Janina Warenholt
Anna Isinger

Background and Objectives. The diagnosis of T-cell neoplasia requires the use of immunohistochemistry on tumor sections or molecular genetic analysis of T-cell receptor (TCR) clonality. Multiplex polymerase chain reactions (PCR) offer a sensitive and expeditious approach to determining clonality early in the diagnostic work-up. We determined the sensitivity and specificity of four multiple PCR for genotyping lymphoid neoplasms at the TCR loci γ (*TCRG*), δ (*TCRD*) and β (*TCRB*, including complete [Vβ-Jβ] and incomplete [Dβ-Jβ] rearrangements).

Design and Methods. Template DNA was derived from frozen or formalin-fixed tissue and from imprints of aspirates or cut tissue surface on a FTA MicroCard® . Each multiplex PCR was performed for 36 cycles in a single tube with multiple previously reported fluorescently labeled primers (*TCRG* and *TCRD*) or novel homologous primers (*TCRB*) and analyzed on electropherograms (Genescan®), applying stringent criteria for interpreting clonal peaks. Two hundred and eleven clinically and immunohistochemically well-characterized benign and malignant non-T-cell lymphoid proliferations, including 138 B-cell lymphomas, were analyzed to determine specificity. The results were compared with those of 28 peripheral and immature T-cell neoplasms and two NK/T-cell lymphomas to determine sensitivity and compute predictive values.

Results. In all T-cell tumors, one or more TCR loci showed clonal rearrangement, which was not evident in two NK/T-cell lymphomas. *TCRG* was the single most informative locus (clonal rearrangement in 89%), followed by *TCRB* (79%) and *TCRD* (39%). Multiplex PCR targeting of *TCRG* and *TCRD* together resolved clonality in all T-cell neoplasms, whereas the *TCRB* locus was clonal in two of three cases with polyclonal *TCRG*. Unexpectedly, in B-cell lymphomas single clonal incomplete *TCRB* (Dβ-Jβ) peaks were 20 times more likely to occur than clonal *TCRG*.

Interpretation and Conclusions. Clonality can be accurately determined in nodal T-cell lymphoma with two single-tube multiplex PCR targeting *TCRG* and *TCRD*. *TCRB* analysis should be considered in equivocal cases in which a polyclonal background may obscure clonal *TCRD*, but clonal incomplete *TCRB* rearrangement alone is insufficient for presuming T-cell lineage. In the absence of objective evidence of B-cell neoplasia, multiplex PCR of T-cell receptor genes may be used early in the diagnostic work-up, including for fine needle aspirates.

Key words: T-cell receptor, clonality, lymphoma, genotype, polymerase chain reaction

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From the Departments of Pathology (MD, JW) and Oncology (AI), Lund University Hospital, Lund, Sweden

Correspondence:
Dr. Michael Dictor, Department of Pathology, Sölvegatan 25, SE 22185 Lund University Hospital, Lund, Sweden.
E-mail: michael.dictor@pat.lu.se

T-cell lymphomas account for at least 10% of lymphoid malignancies, but distinguishing certain T-cell neoplasms from B-cell neoplasms and non-neoplastic T-cell proliferations can be challenging. Rearrangements of T-cell receptor (TCR) loci can be detected by Southern hybridization, which requires abundant high-molecular lymphocyte DNA, is work-intensive and not applicable in all clinical situations. More importantly, the clinical usefulness of showing that a T-cell proliferation is clonal increases if the evidence is found early in the diagnostic work-up. Recent refinements in the detection of clonal rearrangements of T-cell receptor loci γ, δ and β (*TCRG*, *TCRD* and *TCRB*, respectively) using multiplex polymerase chain reactions (PCR) with high resolution fragment detection (Genescan®) offer a sensitive and rapid means of assessing the nature of T-cell proliferations using small amounts of template.[1,2] We evaluated and adapted multiplex PCR procedures, in addition to devising and testing homologous primers for *TCRB*, in order to determine B and T-cell clonality in fine needle aspirates and fresh excised tissue. A rapid turnaround time and relative ease of use and interpretation were deemed important to allow the hematopathologist to analyze results directly in the appropriate clinical and morphological context. Using DNA from lymph nodes routinely excised for lymphoma diagnostics in a typical population, we therefore defined the multiplex PCR necessary to confirm a clonal T-cell proliferation and determined the sensitivity and specificity of each multiplex PCR.

Design and Methods

The diagnostic specificity of the TCR assays was evaluated in tissues from a series

Table 1. TCR analyses in B-cell lymphoma, benign lymphoid proliferations and nodal metastasis.

Category	Material	N	Fraction with clonal (or borderline clonal) Genescan peaks			
			TCRG	TCRD	TCRB (V_β-D_β-J_β)	TCRB (D_β-J_β)
Thymoma	FTA	6	0/6	0/6	0/6	0/6
Benign node (including 23 hyperplasia, 1 mononucleosis, 1 dermatopathic lymphadenopathy, 4 sarcoidosis)	Fresh	40	0/40	0/5	0/5	0/5
Hodgkin's lymphoma						
Classic	Fresh	16	0/16	0/1	0/1	0/1
Nodular lymphocyte predominant	Fresh	1	0/1	ND	ND	ND
Carcinomatous metastasis	Fresh	9	0/9	0/1	0/1	0/1
Diffuse large B-cell lymphoma	Fresh	55	1 (2)/55*	0/11	0/11	2/11*
Follicular lymphoma	Fresh	39	(1)/39	0/9	0/9	2/9
Chronic lymphatic leukemia/small lymphocytic lymphoma	Fresh	14	0/14	ND	ND	ND
Mantle cell lymphoma	Fresh	23	0/23	ND	ND	ND
Other low-grade BCL	Fresh	5	0/5	ND	ND	ND
B-lymphoblastic lymphoma	FTA	1	1/1	0/1	1/1	0/1
T-cell rich B-cell lymphoma	Fresh	2	0/2	ND	ND	ND
Total		**211**	**2 (3)/211**	**0/34**	**1/34**	**4/34**

*FTA, see Design and Methods. Borderline clonal indicates cases (in parentheses) with a dominant peak ~2.2 to 2.4 higher than surrounding peaks. *Includes a single case of DLBCL with clonality for both TCRG and TCRB D_β-J_β. ND, not done.*

of 211 cases of non-T-cell lymphoma, including 139 B-cell lymphomas, 17 Hodgkin's lymphomas, 40 benign lymph nodes, 6 thymomas and 9 nodal carcinomatous metastases. All were assayed in one (n=177) or all four (n=34) multiplex PCR, as shown in Table 1.

The diagnostic sensitivity of each TCR assay was estimated from 30 consecutive cases of T-cell neoplasia, including eight T-lymphoblastic lymphomas, five anaplastic large cell lymphomas (including one primary cutaneous anaplastic large cell lymphoma), nine peripheral T-cell lymphomas, three specimens of mycosis fungoides, two NK/T-cell lymphomas and one each of T-prolymphocytic leukemia, angioimmunoblastic lymphoma and primary cutaneous T-cell lymphoma.[3] All lymphomas were classified according to the recent WHO criteria[4] or proposed WHO-EORTC criteria for primary cutaneous lymphoma[3] after morphological assessment and immunophenotyping with a panel of markers, which included CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD23, CD45RO, CD30, CD45, CD20 and, as needed, CD1a, CD79a, CD21, CD56, TdT (terminal deoxynucleotidyl transferase), CD99, granzyme B, TIA-1 (T-cell intracellular antigen), EMA (epithelial membrane antigen) and ALK-1 (anaplastic lymphoma kinase). The patients' age, sex and tumor genotype are presented in Table 2 and immunophenotypes are shown in Table 3.

Template DNA was either extracted from frozen lymph node tissue with phenol/chloroform followed by precipitation in sodium acetate/ethanol or purified on a column membrane with chaotropic salt (GL Universal DNA Minispin, #D-020-50) or derived (six cases) from 3×10 μm thick paraffin sections treated for at least 3 h in a proteinase K digestion buffer at 56°C without subsequent purification. As a fourth alternative, all six thymomas in Table 1 and nine specimens in Table 2, including one fine needle aspirate, were imprinted or ejected onto solid supports, as detailed below.

Solid matrix support for the DNA template

FTA MicroCard™ (Whatman, #WB120211) is a chemically impregnated solid matrix support that lyses cells, as indicated by a color change in the area of contact, and permanently binds their nucleic acids for indefinite storage at room temperature. A punch 1.2 mm in diameter (or 2-3 punches for a 50 μL PCR reaction) is rinsed 15 min in a purification reagent (Whatman #WB120204) to remove heme and other contaminants, then rinsed 10 min in 10 mM TE buffer (Tris-HCL with 0.1 mM EDTA, pH 8.0). After drying, the punch is placed in a microfuge tube with the PCR reactants. During the PCR, DNA remains bound to the matrix and the punch (if removed promptly after the

Table 2. Specimens, clinical data and Genescan results in 30 T and NK/T-cell neoplasms.*

Case	Material	Age (yrs)/ Sex	Diagnosis	TCRD†	TCRG	TCRBD$_{\beta}$-J$_{\beta}$‡,4	TCRB V$_{\beta}$-D$_{\beta}$-J$_{\beta}$‡
1.	Fresh	62 M	AIL	Polyclonal	Clonal biallelic	Clonal Dβ2-Jβ, Dβ1-Jβ	Polyclonal
2.	Fresh	38 M	ALCL, null cell	Flat line	Clonal biallelic	Polyclonal	Clonal
3.	Fresh	30 M	ALCL, T-cell	Polyclonal	Clonal monoallelic	Clonal Dβ2-Jβ	Polyclonal
4.	Fresh	19 F	ALCL, T-cell	Clonal Dδ2-Jδ, Vδ-Jδ	Clonal biallelic	Polyclonal	Clonal
5.	FTA	58 M	ALCL, T-cell	Clonal Vδ-Jδ	Clonal biallelic	Clonal Dβ2-Jβ, Dβ1-Jβ	Polyclonal
6.	FTA	91 M	ALCL-cutaneous	Flat line	Clonal biallelic	Clonal Dβ2-Jβ	Clonal
7.	Fresh	75 M	MF	Clonal Dδ-Jδ	Clonal biallelic	Clonal Dβ2-Jβ, Dβ1-Jβ	Clonal
8.	FTA	78 M	MF	Flat line	Clonal biallelic	Clonal Dβ1-Jβ	Clonal
9.	FTA, FNA	same	MF, nodal	Flat line	Clonal biallelic	Clonal Dβ1-Jβ	Clonal
10.	Fresh	85 M	PTCL	Clonal Dδ-Jδ	Polyclonal	Clonal Dβ2-Jβ	Polyclonal
11.	FTA	82 M	PTCL	Polyclonal	Clonal biallelic	Polyclonal	Polyclonal
12.	Fresh	67 M	PTCL	Polyclonal	Clonal biallelic	Clonal Dβ2-Jβ	Polyclonal
13.	Fresh	65 M	PTCL	Polyclonal	Clonal biallelic	Clonal Dv2-Jβ	Clonal
14.	FTA	63 F	PTCL	Polyclonal	Clonal biallelic	Polyclonal	Polyclonal
15.	Paraffin	58 F	PTCL	Clonal Vδ-Jδ	Polyclonal	Flat line**	Polyclonal
16.	Fresh	57 M	PTCL	Clonal Dδ-Jδ	Clonal biallelic	Polyclonal	Polyclonal
17.	Fresh	73 F	PTCL	Clonal Dδ-Jδ	Polyclonal	Clonal Dβ2-Jβ	Polyclonal
18.	Paraffin	75 M	PTCL	Clonal Vδ-Jδ	Clonal monoallelic	Clonal Dβ2-Jβ	Polyclonal
19.	Paraffin	68 M	Cutaneous TCL§	Flat line	Clonal biallelic	Clonal Dβ2-Jβ, Dβ1-Jβ	Clonal
20.	Fresh	57 M	T-PLL	Polyclonal	Clonal monoallelic	Clonal Dβ2-Jβ	Polyclonal
21.	Fresh	37 F	TLL	Flat line	Clonal monoallelic	Polyclonal	Clonal x2 (both HEX)
22.	FTA	61 M	TLL	Clonal Dδ-Jδ	Clonal biallelic	Polyclonal	Polyclonal
23.	Fresh	46 M	TLL	Clonal Vδ-Jδ	Clonal monoallelic	Clonal Dβ2-β	Clonal
24.	Fresh	26 M	TLL	Clonal Dδ-Jδ, Vδ-Jδ	Clonal monoallelic	Clonal Dβ2-Jβ	Clonal
25.	Fresh	17 M	TLL	Flat line	Clonal biallelic	Clonal Dβ2-Jβ	Clonal
26.	Paraffin	15 M	TLL	Flat line	Clonal biallelic	Polyclonal	Clonal
27.	Paraffin	1 M	TLL	Clonal Dδ-Jδ, Vδ-Jδ	Clonal monoallelic	Clonal Dβ2-Jβ	Clonal
28.	Paraffin	14 M	TLL	Clonal Dδ-Jδ, Vδ-Jδ	Clonal monoallelic	Clonal Dβ2-Jβ	Polyclonal
29.	FTA	64 F	NK/T, nasal type	Polyclonal	Polyclonal	ND	ND
30.	FTA	58 F	NK/T, nasal type	Polyclonal	Polyclonal	Polyclonal	Polyclonal
Values to the right reflect usefulness of each clonal TCR locus for diagnosing mature T-cell neoplasia, specifically, and do not take into account frequent lineage infidelity in B-lymphoblastic lymphoma.			Sensitivity (95% CI)§§	0.39 (0.22 to 0.59)	0.89 (0.72 to 0.98)	0.68 (0.48 to 0.84)	0.50 (0.31 to 0.69)
			Specificity (95% CI)	1.00 (0.83 to 1.00)	0.99 (0.96 to 1.00)	0.80 (0.56 to 0.94)	1.00 (0.83 to 1.00)
			Positive predictive value (95% CI)	1.00 (0.72 to 1.00)	0.96 (0.80 to 1.00)	0.83 (0.61 to 0.95)	1.00 (0.77 to 1.00)
			Negative predictive value (95% CI)	0.54 (0.37 to 0.70)	0.98 (0.94 to 1.00)	0.64 (0.42 to 0.82)	0.59 (0.41 to 0.75)

*For explanation of FTA, see Design and Methods. ND, not done. AIL, angioimmunoblastic lymphoma; ALCL, anaplastic large cell lymphoma; MF, mycosis fungoides: PTCL, peripheral T-cell lymphoma; TLL, precursor cell T-lymphoblastic lymphoma. *5 cases also showed cross-lineage clonal rearrangement of IGH: 3 cases of TLL and 2 of ALCL. **Flat line suggests either retention of a germline configuration or locus deletion. †Flat line indicates deletion of both TCRD alleles rather than Dδ2-Dδ3 rearrangement, which would have been detected by the green-labeled Dδ3 primer (see Results). ‡Includes examples of HEX-labeled and FAM-labeled clonal products (see Table 4 for J, labeling). §Recurrent nodule on external ear. §§Values in the last four rows are derived from data in Tables 1 and 2.*

PCR and rinsed in buffers) can be reused in up to three consecutive multiplex PCR with different primers for as long as 3 days after the initial reaction. There are two potential problems when using paucicellular material, such as is often present in fine needle aspirates (aspirates may contain from very few up to about 30,000 cells), for clonality detection: template DNA may not suffice for several separate PCR reactions, and small samples increase the risk of pseudoclonality. We deposited an aspirate directly on the FTA support and successfully carried out three consecutive multiplex PCR using the same punch. This method may thus allow TCR rearrangements to be resolved using limited cellular material from a single aspirate containing an unknown number of tumor cells.

Multiplex PCR

The initial single-tube multiplex PCR targeted rearranged *TCRG*, *IGH*, two B-cell translocations, t(14;18) and t(11;14), in addition to a 352-bp globin fragment for control of amplifiability, according to the protocol of Meier *et al.*[2] Vγ, *IGH* FR1 and FR3 primers were labeled 5' with 6-FAM. The second multiplex PCR utilized the BIOMED-2 primers and protocol (i) for *TCRD* rearrangements (Vδ-Jδ, Dδ-Jδ, Dδ2-Dδ3); Jδ primers were labeled with 6-FAM and D3 labeled with HEX. The remaining two reactions targeted the *TCRB* locus, as detailed below. In interpreting Genescan electropherograms (default parameters), for which BIOMED-2 offered no guidelines, we adopted the rule of Meir *et al.*,[2] bolstered by the experience of others,[5] that

Table 3. T-cell lymphoma immunophenotypes in corresponding paraffin sections.

Case	Diagnosis	CD1a	CD2	CD3	CD4/CD8	CD5	CD7	CD30	CD45RO	CD56	EMA	TIA-1/granzyme B	ALK-1	TdT
1.	AIL	ND	ND	+++	ND	ND	ND	–	+++	ND	ND	ND	ND	ND
2.	ALCL, null cell	ND	ND	–	ND	–	–	+++	++	ND	+++	+++	cytoplasmic	ND
3.	ALCL, T-cell	ND	ND	–	CD4>CD8	ND	ND	+++	–	ND	+	+++	–	ND
4.	ALCL, T-cell	ND	ND	–	ND	ND	ND	+++	++	ND	+	ND	cytoplasmic	ND
5.	ALCL, T-cell	ND	ND	++	ND	ND	ND	+++	++	ND	ND	+++	–	ND
6.	ALCL-cutaneous	ND	ND	+++	ND	ND	ND	+++	+	ND	+	++	ND	ND
7.	MF	ND	ND	+++	CD4>CD8	ND	ND	++	+++	ND	ND	ND	ND	ND
8.	MF	ND	ND	+++	CD4++	ND	ND	+	+++	ND	ND	ND	ND	ND
9.	MF, nodal	ND	ND	+++	CD4++	++	ND	++	ND	ND	ND	ND	–	ND
10.	PTCL	ND	ND	+	ND	ND	ND	+++	++	ND	ND	+	–	ND
11.	PTCL	ND	–	+	CD4++	ND	ND	+++	++	ND	–	–	–	ND
12.	PTCL	ND	ND	+++	CD4++	ND	ND	ND	ND	ND	ND	ND	ND	ND
13.	PTCL	ND	++	+++	CD8++	-	++	–	+++	++	ND	ND	ND	ND
14.	PTCL	ND	ND	+++	ND	+++	ND	–	+++	ND	ND	ND	ND	ND
15.	PTCL	ND	+++	++	CD4>CD8	+++	++	+	+++	–	–	–	ND	ND
16.	PTCL	ND	ND	+++	ND	ND	ND	++	+++	ND	ND	–	–	ND
17.	PTCL	ND	ND	++	CD4++	++	++	+	+++	ND	–	ND	–	ND
18.	PTCL	ND	ND	+++	ND	ND	-	++	+++	ND	–	ND	–	N
19.	Cutaneous TCL	–	+++	+++	CD8>CD4	+++	+	–	++	–	–	+++	ND	ND
20.	T-PLL	–	+++	++	CD8+	ND	+	–	+++	ND	ND	ND	ND	ND
21.	TLL*	++	++	+	CD4=CD8	++	++	ND	ND	ND	ND	ND	ND	+++
22.	TLL	–	++	–	–	ND	+++	ND	++	+	ND	ND	ND	+++
23.	TLL	++	++	+++	CD4=CD8	++	++	ND	ND	ND	ND	ND	ND	+++
24.	TLL	ND	-	+++	–	-	++	ND	++	ND	ND	ND	ND	++
25.	TLL	++	++	+++	CD4=CD8	++	++	ND	+++	ND	ND	ND	ND	+++
26.	TLL	+++	+++	+++	ND	ND	ND	ND	+++	–	ND	ND	ND	+++
27.	TLL	++	++	+++	CD8++	++	++	ND	ND	ND	ND	ND	ND	+++
28.	TLL#	+	+	++	–	–	+++	–	+	++	–	ND	–	–
29.	NK/T§	ND	++	$CD3_S$-	–	–	ND	+	++	++	ND	+++	ND	ND
30.	NK/T§	ND	+	CD3ε+	–	–	ND	–	ND	ND	ND	+++	ND	ND

**Phenotyping was by flow cytometry only; °expressed aberrant CD79a; #clinically and histologically consistent with primary cutaneous CD4-positive small/medium-sized pleomorphic T-cell lymphoma despite CD8 phenotype. §EBER1 was positive by in situ hybridization. -, +, ++, +++ indicate the relative proportion of tumor cells staining (<5%, 10-50%, 50-75%, >75%, respectively).*

a peak could be considered clonal if its fluorescence intensity was at least 2.5 times greater than that of surrounding peaks within the relevant size range. An exception was *TCRD*, for which we required a four-fold greater peak height to avoid false positive interpretation of pseudoclonality for a locus with a limited recombinative repertoire. In addition, a peak for any locus was considered informative only if it spanned no more than approximately one nucleotide.

TCRB assay

The *TCRB* locus contains 65 variable (V_β) gene segments, but only about 46 yield a functional protein.[6] Rearrangement of the various gene segments has been described in detail,[1] and the linearly arranged Vβ segments can become juxtaposed to either of two separate Dβ/Jβ families. Non-functional Vβ-Dβ-Jβ rearrangement can be followed by rearrangement of another Vβ to the second set of Dβ/Jβ and constant gene segments on the same allele; alternatively, non-functional and functional rearrangements may occur on separate alleles. We devised 26 forward and 13 reverse primer sequences to anneal with 100% homology in highly conserved regions of 46 functional V, segments and each Jβ1 and Jβ2 gene segment, respectively (Table 4, *based on Genbank accession L36092*). Three forward primers also showed 100% homology with the predicted Vβ pseudogenes V9S2, V13S7 and V13S4, while significant annealing to the remaining non-functional Vβ segments was not predicted (<90% homology). A primer for Vβ20β the only Vβ segment situated downstream of the Jβ clusters, was omitted when a BLAST search indicated the lack of reported complementary sequences in patient material. *In silico* review (using Vector NTI Advance software) of sequenced *TCRB* rearrangements (Genbank, n=77) allowed us to predict a narrow Vβ-(Dβ)-Jβ size range of about 220 to 246 bp. In addition, it confirmed the known predilection for the Jβ2 family in *TCRB* rearrangements.[7] Because of these two facts, we labeled three Jβ2 family primers with FAM, the remaining four with HEX and split the Jβ1 family evenly between the two fluorochromes in order to increase the sensitivity of monoclonal peak detection against a polyclonal *TCRB* background within the narrow size range. Incomplete *TCRB* (DβJβ) rearrangements were analyzed in a separate multiplex PCR using two forward BIOMED-2 primers, which anneal upstream of Dβ1 and Dβ2,[1] and the reverse Jβ primers in Table 4. Primers are all aliquoted in water in the desired molar proportions and maintained at -20°C.

Despite the large numbers of primers for each multiplex PCR, for example 26 primers in the analysis of Meier *et al*. and 39 for the *TCRB* VJβ-DJβ-Jβ multiplex PCR, the mixtures have remained stable even after numerous freeze-thaw cycles. For extracted DNA, 100-300 ng was used as template, while 1-3 punches were taken from FTA MicroCard imprints. The PCR consumed 2.5 units of a heat-activated thermostabile DNA polymerase (AmpliTaq Gold, Perkin-Elmer) in a 50 μL final volume with 4 pmol of each primer (Table 4), 1.5 mM $MgCl_2$ and 0.8 mM dNTP in Gold Buffer. For the DJβ-Jβ tube, reaction conditions were similar except for the use of 1.25 units of polymerase and 8 pmol of each primer. The PCR was run for 37 cycles, starting at 95°C for 10 min, then cycles at 95°C for 45 sec, 58°C for 45 sec, 72°C for 90 sec and ending at 72°C for 20 min. Products were checked by electrophoresis on a 1.8% agarose gel and a 1 μL sample was diluted 3-5 fold, then mixed with 12 μL of formamide and 0.5 μL of ROX-500 size standard before the sample was denatured at 95°C, cooled and loaded on an ABI 3100 Genetic Analyzer running Genescan® software (Applied Biosystems).

Sensitivity was tested both on pure Jurkat cell DNA in dilutions and Jurkat DNA admixed with 50 ng of polyclonal lymphoid DNA from a benign lymph node.

Statistics

Predictive values with confidence intervals for multiplex PCR were calculated using GraphPad InStat version 3.0, GraphPad Software, San Diego, CA, USA.

Results

Evaluation of TCRB multiplex PCR

For *TCRB* Vβ-Dv-Jβ, the peak height distributions in benign lymphoid specimens varied from about 215 to 244 nt, whereas the amplicon size in thymomas was as small as ~200 and as large as 247 nt, indicating a potential span of about 47 nt in benign T cells. Peaks tended to maintain an in-frame spacing of 3 nt. *TCRB* amplicons in T-cell neoplasms ranged from ~217 to ~247 nt, and a modal peak in the amplicon size distribution curve was seen at 232 nt. Because size reflects the differential effect of drag in the polymer due to fluorochrome labels on primers and size standards, which in our experience underestimates sequence lengths by 3 nt, this latter figure corresponds precisely with 235 nt based on sequences in Genbank. By comparison, BIOMED-2[1] reported a size spread of 39 nt among 17 positive samples. In a single-tube multiplex PCR on extracted T-cell neoplasm DNA, we readily detected clonal Vβ-Dβ-Jβ rearrangements without spurious peaks within the expected size interval. Incomplete rearrangements were also readily detected. The size range for Dβ2-Jβ and Dβ1-Jβ was predicted to be ~14

Table 4. Primers for *TCRB* Vβ and Jβ segments.

Gene segment[6]	*Primer sequence*
V_β7S2	TGGTACAAGCAAAGTGCTAA
V_β7S3	
V_β13S9	TGGTATCGACAAGACCCAGG
V_β13S3	
V_β13S2	
V_β13S1	
V_β13S6	
V_β13S7 pseudo	
V_β13S4 pseudo	
V_β15S1	
V_β14S1	
V_β3S1	
V_β13S5	TGGTATAGACAAGATCTAGG
V_β6S1	TGGTACCGACAAAGCCTGGG
V_β1S1	TGGTACCAACAGAGCCTGGA
V_β22S1	TGGTACAGACAAATCTTGGG
V_β21S1	TGGTACCGGCAGATCCTGGG
V_β21S3	TGGTACCAGCAGATCCTGGG
V_β6S11	TGGTACCGACAGASCCTGGG
V_β6S7	
V_β6S12 pseudo	
V_β6S5	
V_β13S3	
V_β5S6	TGGTACCAACAGGYCCTGGG
V_β5S3	
V_β5S2	
V_β6S3	
V_β5S8	
V_β9S1	TGGTATAAACAGGACTCTAA
V9S2 pseudo	
V_β13S8	TGGTACCGACAGGCCCTGGG
V_β6S4	CGGTATCGACAAGACCCAGG
V_β6S14	TGGTATCAACAGGCCCTGGG
V_β7S1	TGGTACAAGCAGAAAGCTAA
V_β23S1	TGGTACCAGCAGGGTCCAGG
V_β12S2	
V_β12S4	
V_β12S3	TGGTATCGACAAGACCYGGG
V_β21S4	TGGTACCTGCAGAACTTGGG
V_β8S1	
V_β8S2	
V_β8S3	TGGTACAGACAGACCATGAT
V_β16S1	TGGTATCGACGTGTTATGGG
V_β24S1	TGGTACCAACAGACCCCAGG
V_β18S1	TGGTATCGGCAGCTCCCAGA
V_β17S1	TGGTACCGACAGGACCCAGG
V_β2S1	TGGTATCGTCAGTTCCCGAA
V_β11S1	TGGTATCAACAAGATCCAGG
V_β4S1	TGGTACCGTCAGCAACCTGG
J_β1S1a	FAM-TGAGTCTGGTGCCTTGTCCAAA
J_β1S2	FAM-TTAACCTGGTCCCCGAACCGAA
J_β1S3	FAM-TGAGCCAACTTCCCTCTCCAAA
J_β1S4	HEX-AGAGCTGGGTTCCACTGCCAAA
J_β1S5	HEX-AGAGTCGAGTCCCATCACCAAA
J_β1S6	FAM-TGAGCCTGGTCCCGTTCCCAAA
J_β2S1	FAM-TGAGCCGTGTCCCTGGCCCGAA
J_β2S2	HEX-TCAGCCTAGAGCCTTCTCCAAA
J_β2S3	HEX-TCAGCCGGGTGCCTGGGCCAAA
J_β2S4	HEX-AGAGCCGGGTCCCGGCGCCGAA
J_β2S5	HEX-CGAGCCGCGTGCCTGGCCCGAA
J_β2S6	FAM-TCAGCCTGCTGCCGGCCCCGAA
J_β2S7	FAM-TGAGCCTGGTGCCCGGCCCGAA

For V_β-D_β-J_β multiplex PCR all primers were combined in one tube. For D_β-J_β, all J_β primers were combined with BIOMED-2 primers D_β1 GCCAAACAGCCTTACAAAGAC and D_β2 TTTCCAAGCCCCACACAGTC in another tube. 5' ends anneal 10 nt downstream and 14 nt upstream of BIOMED-2 V_β and J_β consensus primers, respectively. Primers homologous to functional segments V_β5S1 and V_β20S1 were omitted, as were primers for 15 presumptive pseudogene segments. Note that V9S2 is a transcribed pseudogene. CG content ranges from 35 to 70% and Tm from 38 to 61°C. All primers were purified by high performance liquid chromatography (MWG, Germany).

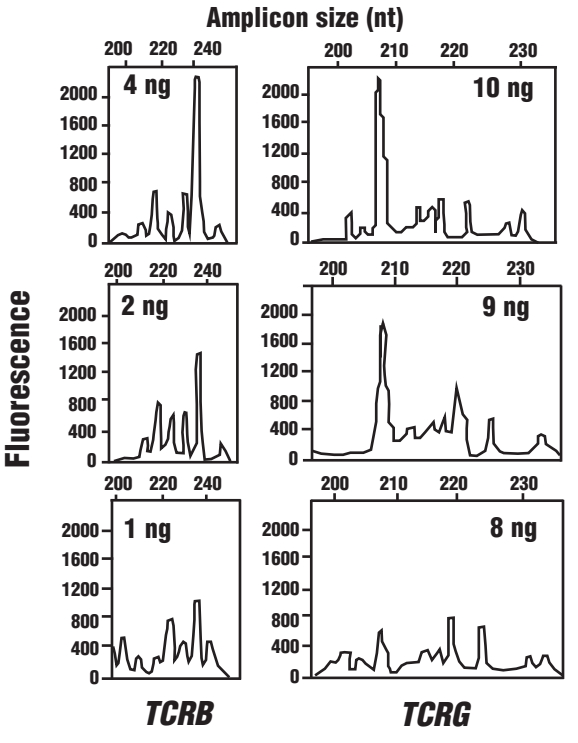

Figure 1. Detection levels for *TCRB* clonality are compared with those of *TCRG* in Jurkat cells (template amount is given in each panel) admixed with 50 ng of DNA from a benign lymph node to produce a polyclonal T/B-cell background. Both loci were amplified in monoallelic peaks, and for the purpose of illustration, the peaks in the top panels are truncated. In the *TCRB* electropherograms, the black curve corresponds to Jβ primers labeled with FAM and the gray curve primers labeled with HEX. Using a cut-off of 2.5 x fluorescence intensity of neighboring peaks, *TCRB* is detectable in 4 ng (4 ng/54 ng, 7% of cellular DNA) as opposed to 10 ng (17%) for *TCRG*, although each clonal peak was still dominant at higher dilutions.

nt shorter than for BIOMED-2, i.e., ~156-196 and ~271-311 nt, respectively, which was confirmed in our analyses.

Sensitivity analysis of the *TCRB* Vβ-Dβ-Jβ assay using diluted Jurkat cell DNA showed a clear-cut Genescan peak at 238 nt with as little as 2 ng of DNA template (equivalent to ~300 cells) in a 50 μL volume. In Jurkat DNA mixed with polyclonal lymphoid DNA, *TCRB* detection sensitivity was compared with that of the *TCRG* multiplex PCR above (Figure 1 truncates the dilution series). Clonal *TCRB* and *TCRG* peaks were clearly detected in as little as 7% and 17% Jurkat DNA/benign DNA mixtures, respectively, while clonal peaks failed to reach the above cut-off threshold as the proportion of Jurkat cell DNA decreased.

Lineage fidelity

As indicated in Table 1 comprising non-T-cell lymphoma lymphoid tissues, the initial multiplex PCR produced clonal rearrangements in one diffuse large B-cell lymphoma (DLBCL) and borderline TCRG electropherograms in two DLBCL and one follicular lymphoma (these four cases also showed B-cell clonality). Thus one could expect at least a suggestive *TCRG* curve in about 3% of B-cell lymphomas, but not in (abundant) benign lymphoid tissue. Neither *TCRD* nor *TCRB* Vβ-Dβ-Jβ showed clonality in 34 samples without T-cell neoplasia with the exception of a single case of B-lymphoblastic lymphoma, a tumor with a well-documented propensity for lineage cross-over.[8] Unexpectedly, *TCRB* Dβ-Jβ analysis produced a single large clonal peak of varying length in four of these 34 samples (12%), including two each of *DLBCL* and follicular lymphoma. One of the *DLBCL* was also clonal for *TCRG*. Thus given our threshold for detection, in these two categories of B-cell lymphoma *TCRB* Dβ-Jβ appeared ~20 times more likely to show cross-lineage clonality than *TCRG* (4/20 vs 1/94; $p<0.005$, Fisher's exact test, two-tailed).

TCR clonality

Results in T-cell neoplasms are summarized in Table 2. Sensitivity, specificity and predictive values of each clonal TCR for T-cell neoplasia, i.e., T-lineage-specific genotyping, based on the contents of Tables 1 and 2, are given at the bottom of Table 2. These aggregate calculations partly reflect the frequency in diagnostic practice of the commonest B-cell lymphomas and may not accurately reflect the expectations for any single category, particularly for rare entities such as B-lymphoblastic lymphoma (which moreover was not included in the calculations). The gene loci combined to produce different patterns of clonality (partly illustrated in Figure 2), which reflected varying assay sensitivity, the proportion of admixed benign T cells and possibly disturbances in the orderly rearrangement of *TCR* loci caused by malignant transformation in precursor cell neoplasms. Twenty-eight cases were true T-cell proliferations, and all carried at least one clonal rearrangement in a TCR locus; 26 specimens (93%) showed clonal rearrangements at two or more loci. Both cases of NK/T-cell lymphoma were polyclonal at all loci tested. Because NK cells characteristically maintain a germline configuration in TCR loci, which are not therefore amplified in conventional PCR, the polyclonal patterns presumably reflected an admixture of reactive T cells in the tumors. Table 5 compares the frequency of clonality in our analyses with those of BIOMED-2, which was similar for *TCRG* and *TCRB* despite our use of different primers and fewer multiplex PCR.

Discussion

Two approaches with PCR were consolidated and refined: the first was reported by Meier *et al.*[2] and detected both B and T-cell clonality in a single multi-

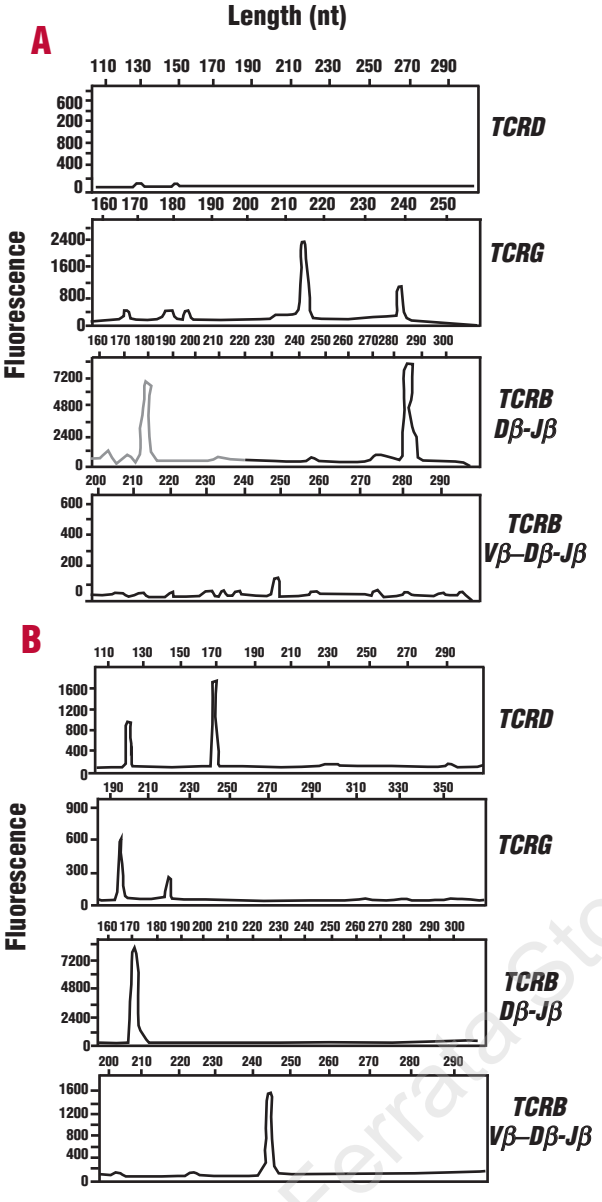

Figure 2. Electropherograms for each TCR locus correspond to row labels on the right. Black curves represent FAM-labeled products, gray curves the HEX label. Column A is case 19 with recurrence of a solitary nodule on the outer ear 10 years after the initial excision. The flat *TCRD* suggests deletion of both alleles, which together with biallelic clonality for *TCRG* (peaks within predicted size range of 180-280 nt) and clonal complete and incomplete *TCRB* rearrangement (peaks implicating both Dβ2-Jβ and Dβ1-Jβ rearrangements) implies *TCRA* rearrangement. Column B is case 27, T-lymphoblastic lymphoma, with biallelic clonal *TCRD* (first peak is within the size range for Dδ2-Jδ rearrangement, ~130-150 nt; second peak corresponds to Vδ-Dδ-Jδ, see table 20 in ref[1] and clonal monoallelic *TCRG*, in addition to clonal complete and incomplete *TCRB* rearrangements (the peak in *TCRB* Vβ-Dβ-Jβ is truncated).

Table 5. Comparison of multiplex PCR/Genescan-determined T-cell neoplasm clonality with BIOMED-2 (1) results.

	*Clonality in T-cell neoplasms**	*BIOMED-2 Clonality in T-cell neoplasms§*	*BIOMED-2 Concordance with clonal Southern blots*
TCRG	25/28 (89%)	–	16/18 (89%)
TCRD	11/28 (39%)	5/18 (28%)	5/6 (83%)
TCRB Dβ-Jβ	19/28 (68%)		
	Either in 22/28 (79%)	–	Either in 23/29 (79%)†
TCRB Vβ-Dβ-Jβ	14/28 (50%)	–	

**multiplex PCRs, four tubes in total; §multiplex PCRs, six tubes; †spurious peak was reported at 273 bp in BIOMED-2 two-tube Vβ-Dβ-Jβ multiplex PCR.*

plex PCR by amplifying *IGH* and *TCRG* rearrangements, in addition to specific translocations, and the second was partly based on a comprehensive study of rearrangements in T-cell receptor gene loci detailed by van Dongen *et al.*[1] for the BIOMED-2 group. The latter defined six separate multiplex PCR (two for *TCRG*, three for *TCRB* and one for *TCRD*) as roughly equal or superior to Southern hybridization in regard to sensitivity and applicability in clinical diagnosis.

By using the multiplex PCR of Meier *et al.* in the initial analysis, adopting the BIOMED-2 protocol for *TCRD* and designing completely homologous Vβ and Jβ primers for *TCRB*, we could reduce the number of multiplex PCR for a complete analysis of amplifiable TCR loci to four, while confirming one or more clonal TCR rearrangements in each of 28 immature and post-thymic T-cell neoplasms using one multiplex PCR each for *TCRG* and *TCRD*. Furthermore, minimal cellular material such as a fine needle aspirate and FTA imprints of cut tumor surface produced clear-cut results, potentially placing molecular analysis at the start of a diagnostic work-up rather than at the tail end when its additive value in diagnosis and treatment planning is often questionable. However, mixing experiments targeting *TCRB* and *TCRG* suggest a level of sensitivity unsuitable for detecting minimal residual disease.

The high frequency of clonal rearrangement in the *TCRG* locus (89%) and its high negative predictive value made this the single most informative locus for T-cell clonality. Sensitivity was in line with the results of BIOMED-2 and exceeded or conformed with commonly reported sensitivities for *TCRG* in PCR.[9-12] The three peripheral T-cell lymphomas with polyclonal *TCRG* each displayed clonal rearrangement in the *TCRD* locus, and two of these also had partial rearrangement (Dβ-Jβ) of *TCRB*. The apparent propensity of the latter for cross-lineage occurrence precludes using it alone to determine T-cell lineage and we have yet to encounter a T-cell lymphoma characterized solely by clonal Dβ-Jβ. Prior work using Southern blots has indicated that mature B-cell lymphomas occasionally (<5%) show cross-lineage

clonality at the *TCRG* locus,[8] but the unexpected higher frequency of clonal Dβ-Jβ in the multiplex PCR does not necessarily indicate that the B cells themselves harbor the rearrangement. Amplification of lymphocyte DNA may detect oligoclones of admixed reactive T cells,[13] although the simultaneous occurrence of multiple high peaks for Dβ-Jβ or *TCRG,* which would have supported this possibility, was not found.

Because both *TCRG* and *TCRD* loci offer a limited repertoire of V-D-J combinations compared with the large diversity inherent in the *TCRB* locus, and γ/δ T-cells show unequal utilization of V-segments in certain tissue compartments, the risk of producing pseudoclonality in cell-poor samples is theoretically greater, although we have yet to experience this. If *TCRG* and *TCRD* analyses are equivocal, *TCRB* may still offer confirmation of clonality. In the T-cell neoplasms clonal Vβ-Dβ-Jβ rearrangement was detected in only half of cases, Dβ-Jβ in over two-thirds and either one in almost 80%, making *TCRB* the second most informative locus. Partial rearrangements may occur together with a complete Vβ-Dβ-Jβ rearrangement on the same or separate alleles,[14] and in ten instances clonal peaks for both types of rearrangement were present. The remaining *TCRB* electropherograms were polyclonal.

A reported validation of the BIOMED-2 *TCRB* protocol (consensus primers) indicated a sensitivity of up to 100% among 102 samples, comprising predominantly T-cell leukemias and only two cases of T-cell lymphomas.[15] Limiting our material in a similar manner would have yielded comparable levels of sensitivity with homologous primers. Because the great majority of peripheral T-cell lymphomas are known to harbor clonal Vβ-Dβ-Jβ and in addition express α/β chains at a four-fold greater frequency than γ/δ,[16] the reason for the reduced sensitivity for complete TCRB rearrangements, with a nearly consistent lack (except for a single case) of clonal Vβ-Dβ-Jβ in peripheral T-cell lymphomas, is unclear. Lowered sensitivity due to background DNA is an unlikely explanation given the 7% threshold of *TCRB* detection. We suggest rather that peripheral T-cell lymphomas may preferentially utilize gene segments which were poorly amplified under the reported conditions.

TCRD was in isolation the least informative locus and was clonally rearranged in only 39%, but had in common with *TCRG* and *TCRB* Vβ-Dβ-Jβ a high specificity for T-cell neoplasia (Table 1). The remainder of the *TCRD* two-color electropherograms were characterized either by a polyclonal curve (32%) or a flat line (29%), the latter consistent with maintenance of germline configuration or locus deletion due to biallelic rearrangement of *TCRA*.

A polyclonal *TCRD* may be partly the result of applying more stringent criteria for interpreting clonality than for the other loci and may indicate the overriding presence of a background of reactive T cells, as illustrated in the two cases of NK/T-cell lymphoma, which in nasopharyngeal specimens is admixed with benign T cells. In this entity, *TCRD* is usually in a germline configuration and thus not amplifiable in routine PCR, but both cases showed a polyclonal pattern rather than a flat curve. Similarly, post-thymic T-cell lymphoma more often than lymphoblastic lymphoma is intermixed with significant numbers of polyclonal T-cells. A flat *TCRD* curve was found only in specimens showing clonal Vβ-Dβ-Jβ rearrangement, including several T-lymphoblastic lymphomas and proportionately fewer mature (post-thymic) T-cell neoplasms (Figure 2), but was less common than the number expected to harbor biallelic *TCRD* deletion.[1]

Clonality assessment and the assignment of cell lineage requires close integration of results with the clinical and morphological details in each case. We recommend an initial multiplex PCR for the *TCRG* and *IGH* loci as early as possible in the diagnostic work-up, for example on a fine needle aspirate. This will confirm clonality in up to 90% of T-cell lymphomas (and most B-cell lymphomas)[2] and points to T-cell lineage if *IGH* is polyclonal in the face of clonal *TCRG*. Suspected T-cell infiltrates without a *TCRG* peak may be further analyzed in a single multiplex PCR for *TCRD* or, as a less favorable alternative, two separate multiplex PCR for complete and incomplete *TCRB* rearrangements. Positivity at two or more T-cell loci offers strong presumptive evidence of T-cell lineage, but if only Dβ-Jβ is clonal lineage determination should be deferred until the relevant immunophenotypic markers are studied.

MD was responsible for the diagnostic work-up and devising multiplex primers for TCRB. JW optimized the use of FTA for lymphoma and, in collaboration with AI, applied and tested multiplex PCR with fluorescence capillary electrophoresis. All authors were involved in interpreting the electropherograms.

All tables and figures were created by MD, who also bears primary responsibility for the paper. The authors declare that they have no potential conflicts of interest.

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