Original Article

A Comparative Study of Polymerase Chain Reaction Detection of Clonal T-cell Receptor Gamma Chain Gene Rearrangements Using Polyacrylamide Gel Electrophoresis versus Fluorescence Capillary Electrophoresis

Mark BY Tang,[1] *MRCP (UK), MMed (Int Med), FAMS*, Tina KL Chong,[1] *BSc (Hons)*, Eugene ST Tan,[2] *MBBS*, Yong Jiang Sun,[3] *MD*, Suat Hoon Tan,[1] *MMed (Int Med), Dip RC Path (DMT), FAMS*

Abstract

Introduction: Polymerase chain reaction (PCR)-based molecular techniques are useful adjunctive tools in the diagnosis of cutaneous T-cell lymphomas (CTCL). This study compares the sensitivity of PCR analysis of the T-cell receptor-γ (TCR-γ) gene rearrangements using conventional polyacrylamide gel electrophoresis (PCR-PAGE) and fluorescent capillary electrophoresis (PCR-FCE). Materials and Methods: A total of 22 paraffin blocks were analysed using PCR-PAGE and PCR-FCE. There were 17 cases of mycosis fungoides (MF), 4 cases of non-MF CTCL and 1 case of lymphoblastic leukaemia. Results: Complete agreement was obtained between PCR-PAGE and PCR-FCE in 19 of the 22 cases, giving a concordance rate of 86.4%. PCR-FCE had a higher sensitivity of 77.3%, compared to 63.6% for PCR-PAGE, allowing the detection of 3 additional cases of clonal T-cell rearrangements, which had equivocal or polyclonal bands on PAGE. Two of these 3 cases were in erythrodermic MF patients. PCR-FCE also allowed the detection of matching clones in serial specimens taken from different sites and at different time intervals in patients with MF. However, matching clones from different specimens can be achieved qualitatively in PCR-PAGE by running and comparing these on the same polyacrylamide gel block. Conclusions: Both PCR-PAGE and PCR-FCE are useful in detecting T-cell clones in CTCL, with both methods being comparable in sensitivity and showing a high concordance rate of 86.4%. PCR-FCE has the added advantage of exhibiting semiquantitative properties, which may be important in early or erythrodermic MF cases, but the requirement for sophisticated and costly machinery limits its availability to high-capacity laboratories. The well-established PCR-PAGE method is a suitable alternative in routine clinical applications.

Ann Acad Med Singapore 2008;37:27-31

Key words: Cutaneous T-cell lymphoma, Mycosis fungoides

Introduction

Cutaneous T-cell lymphomas (CTCL) are a heterogeneous group of malignancies of skin-homing T-cells, of which mycosis fungoides (MF) is the most common form.[1] CTCL can be difficult to diagnose, especially in the early stages or in cases with atypical clinical presentation.[2] Immunohistochemical evidence of aberrant expression of T-cell markers can often be equivocal in the early stages, making it difficult to differentiate neoplastic from reactive lymphocytic proliferations.

Polymerase chain reaction (PCR)-based molecular techniques are increasingly used as adjunctive tools in the diagnosis of CTCL. In contrast to reactive T-cell lymphoproliferative diseases, which are predominantly oligoclonal or polyclonal, T-cell lymphomas are clonal expansions of T-cells, each having an identical T-cell receptor (TCR) gamma (γ) gene rearrangements.[3]

The analysis of TCR-γ PCR products by conventional polyacrylamide gel electrophoresis (PCR-PAGE) is qualitative and widely available in clinical laboratories. Currently, fluorescent capillary electrophoresis (PCR-FCE) is considered the gold standard of PCR methods as it can

[1] National Skin Centre, Singapore
[2] Tan Tock Seng Hospital, Singapore
[3] Department of Infectious Diseases, Tan Tock Seng Hospital, Singapore
Address for Correspondence: Dr Tan Suat Hoon, National Skin Centre, 1 Mandalay Road, Singapore 308205.
Email: shtan@nsc.gov.sg

provide semiquantitative results and detect clone-specific PCR products using an automated, high-resolution capillary electrophoresis system to separate and analyse fluorescence-labelled PCR products.

The aim of this study was to compare the sensitivity of TCR-γ PCR analysis using conventional PCR-PAGE and PCR-FCE.

Materials and Methods

Clinical Specimens and Cell Lines

DNA was extracted from 22 paraffin-embedded skin tissue blocks by the phenol-ethanol precipitation method. There were 17 specimens from patients with MF (specimens 2, 5-15, 17-21), 4 specimens from non-MF CTCL patients (specimens 1, 3, 4 and 16) and 1 specimen from a patient with lymphoblastic leukaemia (specimen 22). Within the MF cases, there were 4 skin biopsies that were reported as histologically suspicious or non-diagnostic for MF (specimens 5, 7, 13 and 20), but all of which were later confirmed to have MF based on serial biopsies obtained from other sites (Table 1). The diagnosis of all patients was based on the revised classification for cutaneous lymphomas adopted by the World Health Organization and European Organization for Research and Treatment of Cancer.[4]

PCR Primers and Conditions

PCR DNA amplification was carried out using an automated thermocycler, GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA).

Four TCR-γ V-J junctions were targeted using the following primers: Vγ2, Vγ9, Vγ10 and Vγ11, with all 4 J segments, namely, Jγ2, JγP, JPγ1 and JPγ2[5] (Table 2). For FCE, the same set of Vγ primers were labelled with 6-carboxy-fluorescein. Primers used for PAGE were not fluorescence-labelled.

The final Vγ primer concentration was 0.5 μM and each

Table 1. Comparison of TCR Gene Rearrangement Detection by PCR-PAGE and PCR-FCE in 22 Cases

Specimen	Histological diagnosis	PCR-PAGE	PCR-FCE (Rn)
1	NK/T-cell lymphoma	+	+ (17.0)
2	Plaque-stage MF	+	+ (39.0)
3	Primary cutaneous anaplastic large-cell lymphoma	+	+ (6.1)
4	Lymphomatoid papulosis	+	+ (5.0)
5	Suggestive of MF	+	+ (5.0)
6	Folliculotropic MF	+	+ (8.4)
7	Atypical lymphoid proliferation in patient with folliculotropic MF	+	+ (12.0)
8	Patch-stage MF	+	+ (4.0)
9	Patch-stage MF	+	+ (3.5)
10	Patch-stage MF	+	+ (9.2)
11	Pigmented purpuric-like MF	+	+ (11.0)
12	Plaque-stage MF	+	+ (18.0)
13	Suggestive of MF	+	+ (3.5)
14	Folliculotropic MF	+	+ (3.5)
15	Patch-stage MF	-	-
16	Primary cutaneous anaplastic large-cell lymphoma	-	-
17	Patch-stage MF	-	-
18	Patch-stage MF	-	-
19	Patch-stage MF	+/-	+ (4.5)
20	Subacute dermatitis (in erythrodermic MF)	+/-	+ (4.0)
21	Erythrodermic MF	+/-	+ (3.0)
22	Lymphoblastic leukaemia	Polyclonal	Polyclonal

+ monoclonal population detected; - monoclonal population not detected; +/- equivocal

MF: mycosis fungoides; PCR-FCE: polymerase chain reaction-fluorescent capillary electrophoresis; PCR-PAGE: polymerase chain reaction-polyacrylamide gel electrophoresis; TCR: T-cell receptor

Table 2. TCR-γ Gene Primers for Polymerase Chain Reaction[5]

Primer	Sequence 5' to 3'
Vγ2	FAM-TAC ATC CAC TGG TAC CTA CAC CAG
Vγ9	FAM-GAA AGG AAT CTG GCA TTC CGT CAG
Vγ10	FAM-AAG CAA CAA AGT GGA GGC AAG AAA G
Vγ11	FAM-AGT AAA AAT GCT CAC ACT TCC ACT TC
Jγ2	TAC CTG TGA CAA CAA GTG TTG TTC
JγP	AAG CTT TGT TCC GGG ACC AAA TAC
JPγ1	GAA GTT ACT ATG AGC TTA GTC CCT T
JPγ2	GAA GTT ACT ATG AGC CTA GTC CCT T

final Jγ primer concentration was 0.3 μM in 50 μL PCR reaction mixture.

In every experiment, 2 separate blank controls were used as negative controls; one introduced at the DNA extraction process (Eppendof tube containing extraction reagents without template DNA and continuing into PCR) and the other at the PCR amplification process (PCR reaction tube containing PCR regents without template DNA). The positive controls used were Vγ2 and Vγ11 from Jurkat cell lines (1:1000), and Vγ9 and Vγ10 from HSB2 cell lines (1:50). A set of primers targeting the CpG island sequence of human genomic DNA (GenBank accession no. AF080684) was used as internal controls to check the quality of extracted DNA and presence of PCR inhibitors from each specimen.

Analysis of PCR products

The analysis of PCR products was performed on PAGE and FCE in parallel experiments by the same technician.

Polyacrylamide gel electrophoresis: PAGE was performed using standard protocol.[6] Samples (15 μL sample and 1.5 μL loading buffer) were loaded into the wells, and electrophoresed at 100 V for 45 minutes. The gels were transferred into a container containing 100 mL TBE buffer and 5 μL ethidium bromide before being photographed under an ultraviolet transilluminator. Clonal T-cell populations were determined by visual inspection and categorised as follows: monoclonal if one distinct band in a single lane was found (Fig. 1), or polyclonal if more than 2 distinct bands per smear were noted.

Fluorescent capillary electrophoresis: Each PCR product was diluted 5 times prior to FCE in order to minimise the heavy background. FCE was performed using the PRISM 3100-Avant Sequence Analyser (Applied Biosystems). A 1-μL sample was combined with 9.5 μL deionised formamide and 0.5 μL GenScan-500 ROX internal size standard (Applied Biosystems). The mixture was denatured at 95°C for 3 minutes, followed by rapid chilling on ice to produce single-stranded DNA. Each sample was then injected for 3 seconds and electrophoresed at 60°C, 15 kV for approximately 45 minutes using 36-cm capillary containing performance-optimised polymer matrix. Each PCR product was size-separated and its fluorescence signals were converted into an electrophoretic profile generated by GeneScan 3.7 software (Applied Biosystems). The x-axis on each profile corresponds to the PCR product size in base pair while the y-axis represents the peak height of each PCR product. From the electrophoretic profiles (Fig. 1), the predominant monoclonal population was identified by analysing the validated peak height ratio, Rn.[7] The Rn value is taken to be the ratio of a peak of interest to the average of the 2 immediate flanking peaks. A Rn value of ≤1.9 implies the absence of any clonal population, while Rn ≥3.0 is consistent with the presence of a monoclonal population. Rn values that fall between 1.9 and 3.0 are considered within an intermediate range that requires visual examination to determine if the peak should be interpreted as monoclonal.

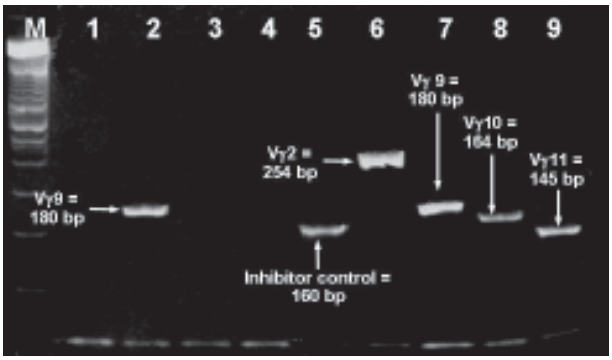
To ensure reproducibility of the PCR results, the entire experiment from DNA extraction to PCR reaction was performed in duplicate for all cases that had initial positive results either by PAGE or FCE PCR.

Results

The results of the TCRγ gene rearrangement analysis using PCR-PAGE and PCR-FCE are summarised in Table 1. Of the 22 cases analysed by PCR-PAGE, 14 cases showed 1 distinct band on visual inspection, consistent with monoclonality. Polyclonal bands were found in 1 case (specimen 22) while 4 specimens (specimens 15 to 18) were negative. PCR-PAGE was equivocal in 3 cases (specimens 19 to 21) exhibiting weakly positive, indistinct bands. Overall, the sensitivity of PCR-PAGE in detecting TCRγ gene rearrangements in this series of patients was 63.6% (14 of 22 cases).

Using PCR-FCE, 17 cases (including 3 cases which had equivocal PCR results on PCR-PAGE) were positive for monoclonality, 4 were negative (specimens 15 to 18) and 1 showed polyclonal peaks (specimen 22). All the negative cases and the polyclonal case by PCR-FCE also showed identical results on PCR-PAGE. Overall, the sensitivity of PCR-FCE in the detection of TCRγ gene rearrangements in this study was 77.3% (17 of 22 cases).

Complete agreement was obtained between PCR-PAGE and PCR-FCE in 19 of 22 cases, giving a concordance rate of 86.4%. Of this, 14 cases with clonal bands on PAGE produced distinct corresponding peaks on FCE, 4 cases (specimens 15 to 18) were negative by both methods, and 1 case (specimen 22) was polyclonal. There were 3 cases



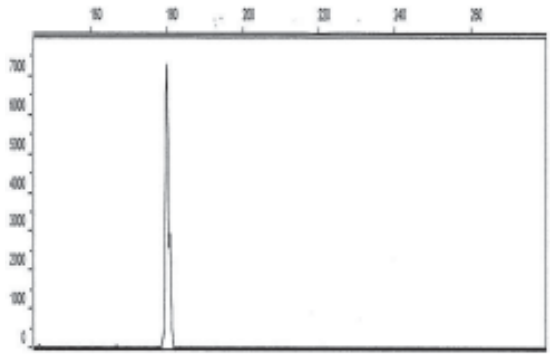
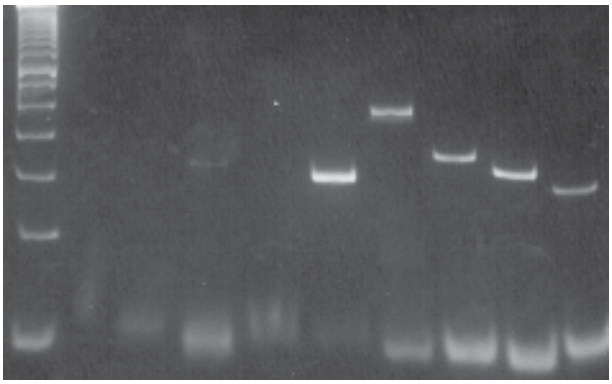
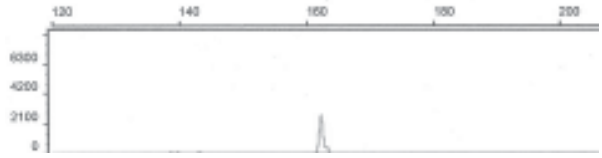


Fig. 1. Specimen 1: PAGE and FCE both showed monoclonal population for Vγ9 gene segment (lane 2 on polyacrylamide gel, left; electrophoretic profile generated by FCE, right).
M = DNA molecular size marker, 50bp
Lane 1-4 = patient's sample; Vγ2, Vγ9, Vγ10, Vγ11 respectively
Lane 5 = internal control
Lane 6-9 = positive control; Vγ2, Vγ9, Vγ10, Vγ11 respectively



*very faint band observed in S-V10 (equivocal +/-) on PAGE



One peak observed on electrophoretic profile by FCE, it is a V10 peak (164 bp)

Fig. 2. Example of a case where PAGE was equivocal but FCE showed clonal rearrangement.
S = sample
PC = Positive control

(specimens 19 to 21) with equivocal results on PAGE but distinct clonal peaks on FCE (Rn 4.5, 4.0 and 3.0 respectively), giving a disconcordance rate of 13.6% (Fig. 2). Of note, 2 of these 3 cases occurred in patients with erythrodermic MF.

In addition, 17 additional specimens taken from within the same group of 22 cases were analysed with FCE (results not shown).These were serial biopsies or multiple biopsies taken from different sites obtained from 6 patients with MF. Running PCR products from different specimens on the same gel, we were able to demonstrate identical T-cell clones in these serial specimens.

Discussion

In our study, comparison of PCR-PAGE and PCR-FCE showed a good concordance rate of 86.4%. This is similar to the findings by Luo et al,[7] who reported a concordance rate of 86%.

Although PCR-PAGE showed an overall acceptable sensitivity of 63.6% in this study and has been shown to be a sensitive method for detecting clonal T-cell populations in early MF,[8] it has several noteworthy limitations. First, PCR-PAGE may be less accurate, especially in cases with a polyclonal background or when clonal T-cells form less than 0.1% of the infiltrate.[5] This was evident in this study, where 3 cases with equivocal bands on PAGE were clearly monoclonal by FCE. As highlighted, 2 of these cases occurred in erythrodermic MF patients. This is significant since erythrodermic MF is known to be difficult to diagnose histologically, and often requires the support of ancillary investigations such as molecular genetic analysis for T-cell clonality.[9]

There is also a limited separation capacity of PAGE, which relies on DNA separation based on molecular size rather than nucleotide sequence. This is of particular importance in TCRγ gene rearrangement analysis because of the limited heterogeneity of the TCRγ locus,[10] resulting in a smaller variability in the lengths of the different rearrangements, which may not be optimally analysed by PAGE. Thus, only clonal fragments of sufficient size and sequence differences can be visually detected against the background population.[7] Another requirement of PCR-

PAGE is the need for skilled and trained personnel for the interpretation of qualitative PCR results.

PCR-FCE is currently considered the gold standard of PCR methods as it makes use of objective, semiquantitative criteria to establish T-cell clonality, thus increasing the sensitivity and reliability of this method.[11] It uses a high-resolution capillary electrophoresis system, together with a computerised data processing and analysis software system that is fully automated, with a fast processing time. This study found a sensitivity of 77.3% for FCE, comparable to the 90% detection rate reported by Luo et al.[7] Another distinct advantage of FCE is that it enables highly precise determination of PCR product size, allowing the detection of clone-specific PCR products. In this study, FCE allowed the accurate detection and matching of identical clones in multiple specimens taken from different sites and at various time intervals in 6 patients with MF. This finding of matching clones from different sites in the same patient adds significant weight to the diagnosis of CTCL, especially in cases of early MF. This study also suggests that PCR-FCE may be superior to PCR-PAGE in detecting monoclonality in patients with erythrodermic MF.

Conclusions

Both PCR-PAGE and PCR-FCE are useful in detecting T-cell clones in CTCL, with both methods showing a high concordance rate of 86.4%. PCR-FCE was superior in cases where PCR-PAGE showed equivocal bands, and is potentially useful as an ancillary investigation for the diagnosis of erythrodermic MF. In addition, PCR-FCE was able to objectively identify the same matching clones in serial biopsies of patients obtained from different sites or at different times. However, PCR-FCE requires sophisticated and costly equipment, which limits its availability to only higher-capacity laboratories. In this instance, PCR-PAGE remains a well-established viable alternative for routine clinical applications, as demonstrated in this study.

Acknowledgements

We would like to thank Dr Christian Sander and Ms Ursula Puchta, Department of Dermatology, Ludwig Maximilians Universität München, Munich, Germany for providing us with HSB2 clonal T-cell lines. Ms Jacqueline Ong, Laboratory Technician, National Skin Centre, Singapore for her help in this project. This project was supported by the National Medical Research Council Enabling Grant FY2003 (R03-06).

REFERENCES

1. Willemze R. Cutaneous T-cell lymphoma: epidemiology, etiology, and classification. Leuk Lymphoma 2003;44(Suppl):S49-S54.
2. Pimpinelli N, Olsen EA, Santucci M, Vonderheid E, Haeffner AC, Stevens S, et al; International Society for Cutaneous Lymphoma. Defining early mycosis fungoides. J Am Acad Dermatol 2005;53:1053-63.
3. Muche JM, Lukowsky A, Asadullah K, Gellrich S, Sterry W. Demonstration of frequent occurrence of clonal T-cells in the peripheral blood of patients with primary cutaneous T-cell lymphoma. Blood 1997;90:1636-42.
4. Willemze R, Jaffe ES, Burg G, Cerroni L, Berti E, Swerdlow SH, et al. WHO-EORTC classification for cutaneous lymphomas. Blood 2005;105:3768-85.
5. Greiner TC, Raffeld M, Lutz C, Dick F, Jaffe ES. Analysis of T-cell receptor gene rearrangements by denaturing gradient gel electrophoresis of GC-clamped polymerase chain reaction products. Am J Pathol 1995;146:46-55.
6. Detection of DNA in polyacrylamide gel by staining. In: Sambrook J, Russell DW, editors. Molecular Cloning: A Laboratory Manual. Vol. 1, 3rd ed. New York: Cold Spring Harbor Laboratory Press, 2001.
7. Luo V, Lessin SR, Wilson RB, Rennert H, Tozer C, Benoit B, et al. Detection of clonal T-cell receptor ã gene rearrangements using fluorescent-based PCR and automated high-resolution capillary electrophoresis. Mol Diagn 2001;6:169-79.
8. Curco N, Servitje O, Llucia M, Bertran J, Limon A, Carmona M, et al. Genotypic analysis of cutaneous T-cell lymphoma: a comparative study of Southern blot analysis with polymerase chain reaction amplification of the T-cell receptor-gamma gene. Br J Dermatol 1997;137:673-9.
9. Vonderheid EC. On the diagnosis of erythrodermic cutaneous T-cell lymphoma. J Cutan Pathol 2006;33(Suppl):27-42.
10. Lefranc MP, Chuchana P, Dariavach P, Nguyen C, Huck S, Brockly F, et al. Molecular mapping of the human T cell receptor gamma (TRG) genes and linkage of the variable and constant regions. Eur J Immunol 1989;19:989-94.
11. Sprouse JT, Werling R, Hanke D, Lakey C, McDonnel L, Wood BL, et al. T-cell clonality determination using polymerase chain reaction (PCR) amplification of the T-cell receptor gamma-chain and capillary electrophoresis of fluorescently labeled PCR products. Am J Clin Pathol 2000;113:838-50.