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Fast molecular diagnostics of canine T-cell lymphoma by PCR and capillary gel electrophoresis with laser-induced fluorescence detector

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Abstract

Lymphoma is the most common hematopoietic tumor in dogs and manifests as a proliferation of malignant lymphoid cells primarily affecting the lymph nodes or solid visceral organs. We describe the use of capillary gel electrophoresis (CGE) with a laser-induced fluorescence (LIF) detector based on polymerase chain reaction (PCR) to rapidly detect a disorder of the canine T-cell receptor γ (TCRγ) gene. After the PCR amplification of the specific TCR(gene in dogs, the 90-bp DNA fragment amplified was separated in a fused-silica capillary by CGE–LIF. Under an electric field of 375 V/cm and with a sieving matrix of 1.5% poly (ethyleneoxide) (M_r 600,000), the amplified PCR products were analyzed within 4 min by CGE separation. When the CGE–LIF method was applied to real clinical samples of the specific DNA fragment of the TCR(gene, the migration time and the corrected peak area showed relative standard deviations ($n=5$) of 0.29% and 0.58%, respectively. Both methods of CGE–LIF and slab gel electrophoresis showed same results for nine clinical samples. This PCR/CGE–LIF technique may prove to be a new fast and simple tool for the rapid diagnosis of the PCR-amplified DNA of canine T-cell lymphoma.

Keywords: Canine T-cell lymphoma; Fast detection; Capillary gel electrophoresis; Polymerase chain reaction

1. Introduction

Lymphoma is the most common hematopoietic neoplasm [1] and the third most common malignant neoplasm in dogs [2]. Canine lymphoma is diagnosed primarily by a cytological assessment of the circulating lymphocytes, cavity fluid, lymphoid tissue or on a histological examination of the lymphoid tissue [3,4]. Madewell [5] defined peripheral blood involvement as the "presence of atypical or immature lymphocytes on blood smears with or without lymphocytosis" and revealed 65% of dogs with multicentric lymphoma to be in stage V. Raskin and Krehbiel [6] defined peripheral blood involvement as cellular atypia and absolute lymphocyte counts (>10,000 ea/μL) and reported that peripheral blood involvement occurred in only 28% of dogs with multicentric lymphoma. However, a specific example of the cytological or histological dilemmas in veterinary medicine includes circulating large granular lymphocytes, which can be observed in both *Ehrlichia canis* (*E. canis*) infections and leukemia [7–9]. A characteristic lymphoma with a wide spectrum of histological appearances and variability in the tumor cell phenotype can make a clinical diagnosis difficult. Therefore, more sensitive and objective assays are needed to detect canine lymphoma.

In the early 1990s, a diagnosis of lymphoma was made by a polymerase chain reaction (PCR) assay because the sensitivity for the aberrant DNA sequences (mutations, rearrangements, deletions and translocations) of lymphoma was very high [1,10–13]. Among these abnormal DNA sequences of lymphoid cells, the antigen receptor gene rearrangement suggests that each segment of variable (V_H), diversity (D_H), joining (J_H) gene rearrange during the development process of lymphoid cells with a specific rearrangement for each lymphocyte [1]. Therefore, most oncogenic complications can be detected by measuring these specific rearrangements. The CDR3 (3rd complementary determining region) of both immunoglobulin and T-cell receptor γ (TCRγ) genes encodes the antigen-binding region of the respective receptor and contains the majority of this unique sequence. In T cells, CDR3 of the TCRγ gene is produced through a recombination of the V and J genes [1,3,4,14–17]. Therefore, a diagnosis of T-cell lymphoma by a PCR assay is very useful

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and has been widely used. However, the process of a PCR assay accompanied with slab gel electrophoresis requires several hours until the result can be available, although it offers the results of high sensitivity. And Southern blotting, which is the standard method for detecting a monoclonal gene rearrangement, requires 1 week [1,11,12].

Recently, due to the labor-intensive, time-consuming nature and requirement of a large sample in traditional slab gel electrophoresis, alternative electrophoretic formats have been developed in the form of capillary electrophoresis (CE) [18]. CE has been used to examine clonality in the lymphoid processes [19–21]. CE has a better resolution efficiency, sensitivity, as well as smaller sample amount and less analysis time than conventional slab gel electrophoresis [22], which allows the separation and identification of PCR products differing in size by only 1 bp [23]. CE also allows a more precise determination of the product size without the problems of band-shift artifacts, and gel-to-gel variations are often associated with agarose or polyacrylamide gel electrophoresis.

In this study, PCR was used to amplify the variable regions of the TCRγ genes in T-cell lymphoma to detect the presence of a clonal lymphocyte population using the primers specific to the conserved regions of the V and J genes to amplify CDR3. Using the CGE technique with a laser-induced fluorescence (LIF) detector, the PCR-amplified specific DNA fragments were analyzed completely within 4 min under an electric field of 375 V/cm and with a sieving matrix of 1.5% poly (ethyleneoxide) (M_r 600,000). This PCR–CGE technique may prove to be a new fast and simple tool for the rapid diagnosis of the PCR-amplified DNA of canine T-cell lymphoma.

2. Experimental

2.1. Chemical and reagents

The 10 × PCR buffer (*w*/20 mM $MgCl_2$), 2.5 mM dNTPs and 5 U *Taq* DNA polymerase were purchased from iNtRon Biotechnology (Daejeon, Korea). A 1 × TE buffer solution (pH 8.0 with Trizma-base) was made from 50 mM Tris–HCl and 2 mM EDTA (Sigma, St. Louis, MO, USA) in deionized water. The dynamic coating matrix of the capillary was made by dissolving 1.0% (w/v) of polyvinylpyrrolidone (PVP, M_r 1,000,000) (Polyscience, Warrington, England) in the 1 × TE buffer together with 0.5 μg/mL ethidium bromide (EtBr, Sigma, St. Louis, MO, USA). The mixture was shaken for 2 min and left to stand for 2 h to remove any bubbles. The sieving matrix was made by dissolving 1.5% (w/v) of poly (ethyleneoxide) (PEO, M_r 600 000) (Sigma, St. Louis, MO, USA) in the 1 × TE buffer along with 0.5 μg/mL EtBr. The matrix was then stirred slowly overnight. A 50-bp DNA ladder (1 μg/μL) (Invitrogen, Carlsbad, CA, USA) was used in the slab gel electrophoresis and CE.

2.2. Clinical sample preparation

The canine T-cell lymphoma samples were acquired from the department of veterinary internal medicine, Chonbuk National University. A 6-year-old neutered male Chihuahua had a history of an enlarged the submaxilla lymph node. The dog was diagnosed with multicentric lymphoma (grade IV) according to the WHO standard, physical examinations and a cytological assessment of the fine needle aspirations. Two milliliters of peripheral blood was obtained by venipuncture, and collected into an ethylenediaminetetraacetic acid (EDTA) anticoagulant tube for a diagnosis of T-cell lymphoma by PCR assay.

Table 1
Primers used to amplify Cμ (positive control) and TCRγ CDR3

Product	Primer names	Primer specificity	Primer sequence (5′-3′)
Cμ	Sigmf1	Cμ	TTC CCC CTC ATC ACC TGT GA
	Srμ3	Cμ	GGT TGT TGA TTG CAC TGA GG
TCRγ	TCRγ1	JH	ACC CTG AGA ATT GTG CCA GG
	TCRγ2	JH	GTT ACT ATA AAC CTG GTA AC
	TCRγ3	VH	TCT GGG A/GTG TAC/T TAC TGT GCT GTC TGG

2.3. PCR sample preparation

The genomic DNA was extracted from the peripheral blood using a GENE ALL™ total DNA extraction kit for blood (Generalbiosystem, Seoul, Korea), according to the manufacturer's instructions. Sigmf1 and Srμ3 primers were used to amplify the Cμ gene as a positive control (Table 1). These PCR primers were designed by Primer Express software v2.0 (Applied Biosystems, Foster, CA, USA). The total volume of the PCR reaction was 50 μL. The PCR mixture contained 5 μL of 50–100 ng template DNA in 5 μL of 2.5 mM deoxynucleoside triphosphate (dNTPs), 5 μL of 10 × PCR buffer (*w*/20 mM $MgCl_2$), each 2 μL of Sigmf1 and Srμ3 primers (10 pmol/μL) and 5U of *i-Taq*™ DNA Polymerase (5U/μL) (iNtRon Biotechnology, Daejeon, Korea).

The TCRγ1, TCRγ2 and TCRγ3 primers were used to amplify the TCRγ gene, (Table 1). These PCR primers were also designed by Primer Express software v2.0. The total volume of the PCR reaction was 50 μL. The PCR mixture contained 5 μL of the 50-100 ng template DNA in 5 μL of 2.5 mM deoxynucleoside triphosphate, 5 μL of 10 × PCR buffer (*w*/20 mM $MgCl_2$), 2 μL of each the TCRγ1, TCRγ2 and TCRγ3 primers (10 pmol/μL) and 5U of *i-Taq*™ DNA Polymerase (5U/μL) (iNtRon Biotechnology, Daejeon, Korea). The PCR was performed in a thermal cycler (MJ Research PTC-200, Waltham, MA, USA) using the following temperature protocol: initial denaturation at 95 °C for 15 min followed by 35 cycles of denaturation at 94 °C for 8 s, annealing at 60 °C for 10 s, extension at 72 °C for 15 s. No final extension time was used [3].

2.4. Slab gel electrophoresis

The amplified DNA was identified by slab gel electrophoresis in 2% agarose gel (Sigma, St. Louis, MO, USA) with 1 × TAE buffer (1.5 M Tris[hydroxymethyl]aminomethane, 1 M acetic

acid and 0.1 M EDTA). Two microliters of 6 × gel loading dye (bromo phenol blue:xylene cyanol FF:glycerol = 0.25:0.25:30, %w/v) was mixed with 10 μL of each specimen. Of this, 12 μL was loaded on 5-well gels and run at 150 V for 60 min in the SaB-Cell (Bio RAD, Hercules, CA, USA). After electrophoresis, the gel was stained with EtBr (0.5 μg/mL) for 10 min and de-stained in nuclease-free water. The samples were then photographed over UV-light of Gel Doc 2000 (Bio RAD, Hercules, CA, USA). The presence of the 90-bp and 130-bp DNA bands was recorded as being positive for T-cell lymphoma and confirmed for canine blood, respectively. The sizes of the DNA product were determined relative to those of a size marker, i.e. the 50-bp DNA ladder.

2.5. Home-built capillary gel electrophoresis with LIF detector

The experimental CGE–LIF setup was similar to that described previously [22]. A He–Ne laser (λ_{ex} = 543 nm; Melles Griot 05-LGR-193, USA) was used as the light source in a home-built CE system, which was coupled to a laser-induced fluorescence detector. A Spellman 1000R high-voltage power supply (Spellman High Voltage Inc., Hauppauge, NY, USA) was used to drive the electrophoresis. A 40-cm total length (20-cm effective length) and a 50-μm I.D. bare fused-silica capillary (Polymicro Technologies Inc., Phoenix, AZ, USA) was used as the separation capillary. Both the coating material and sieving matrix were sequentially injected hydrodynamically at one end of the capillary using a syringe during 2 min and 3 min, respectively. The sample was injected electrokinetically at 75 V/cm for 30 s. Sample separation was then performed in an electric field of 375 V/cm. After each run, the capillary was reconditioned before the subsequent analysis by refilling with sieving matrix for 3 min. The data was recorded as a function of time during the CGE, and was saved on an IBM-compatible computer (1.70-GHZ Pentium IV) at 5 Hz. Data treatment and analysis were performed using an Autochro® data system (Young Lin Instrument Co., Anyang, Korea).

3. Results and discussion

3.1. Identification of the amplified DNA by slab gel electrophoresis

The conventional methodology for detecting the PCR-amplified products of a variable region (V_H) of TCRγ genes in T-cell lymphoma to determine the presence of a clonal lymphocyte population involves electrophoresis on agarose or polyacrylamide gel. Fig. 1 shows the EtBr stained 2.0% agarose gel for the assay of the PCR-amplified DNA fragment of the TCR(gene. The PCR products of the positive DNA control (Cμ) and T-cell lymphoma sample (TCRγ gene) from slab gel electrophoresis analysis are shown in lanes 1 and 3. The Cμ product was approximately 130-bp DNA and the TCR(product centered at approximately 90-bp DNA. A result was considered positive if one or more dominant and discreet clonal bands were present

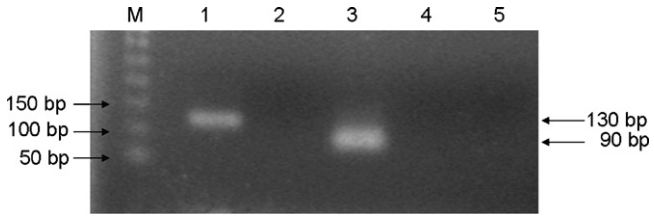

Fig. 1. Slab gel electrophoresis of the PCR-amplified DNA fragment of TCR(gene (90-bp DNA). Lane: (M) DNA marker (50-bp DNA ladder), (1) positive control of dog (Cμ, 130-bp DNA), (2) negative control (cattle blood), (3) T-cell lymphoma sample (TCRγ gene), (4) water control for Cμ primers, (5) water control for TCRγ primers. Gel electrophoresis conditions: 2% agarose gel matrix in 1 × TAE buffer; applied voltage, 150 V for 60 min; ambient temperature.

at irregular intervals. There were no bands at the PCR product of the normal cattle blood (lane 2 in Fig. 1), water controls for the Cμ primers and TCR(primers (lanes 4 and 5 in Fig. 1).

3.2. PCR-based capillary gel electrophoresis with LIF detector

CGE–LIF was examined as an alternative to slab gel electrophoresis for the analysis of PCR-amplified products (90-bp DNA) indicative of a TCRγ gene disorder for diagnosis of canine T-cell lymphoma. Initially, an attempt was made to obtain the optimum CGE condition using the 50-bp DNA ladder. The higher PEO concentrations showed a better resolution between the 50-bp and 100-bp DNA fragments, but required a longer migration time (Table 2). The electric field also affected the resolution and migration time of the PEO gel matrix condition. Using a relatively high electric field, the DNA fragments could be separated within a relatively short time. However, excessively high electric field strength reduced the resolution in the CGE–LIF system (over 375 V/cm at 0.5% PEO in Table 3). Generally, the main separation mechanism in CGE is based on the differences in the DNA size through a gel matrix-filled capillary. Small DNA fragments can pass through the gel and elute first, whereas larger DNA fragments are retarded by the gel and elute later. At an electric field of 375 V/cm and 1.5% PEO, the 50-bp and 100-bp DNA fragments showed a relatively short analysis time with a resolution (R_s) of 3.1 (Table 3), while both DNA fragments were not separated from the baseline at a relative low PEO concentration (≤0.5%) and high electric field strength (≥375 V/cm).

Fig. 2 shows the representative electropherograms of CGE–LIF analysis of the selected samples shown in Fig. 1. In order to determine the CGE optimum separation conditions for the rapid detection of canine T-cell lymphoma, the amplified 90-bp DNA of the specific TCRγ gene and Cμ (130-bp DNA fragment of positive control) by PCR were injected into the PEO gel-filled capillary and the results were compared with those from slab gel electrophoresis. At a constant electric field strength of 375 V/cm, both 90-bp and 130-bp DNA fragments were analyzed successfully within 4 min in a 1.5% PEO (M_r 600,000) sieving matrix and 1 × TE buffer with 0.5 μg/mL EtBr (Fig. 2B–D). The detection of the amplified PCR products in CGE–LIF was much easier, and the procedure was much faster than the conventional slab gel electrophoresis.

Table 2
The migration time of the 50-bp and 100-bp DNA fragments according to the electric field and concentration of the PEO sieving gel by CGE–LIF ($n = 5$)

PEO (%)	Ladder size (bp)	Electric field (V/cm)				
		250	312.5	375	437.5	500
0.5	50	4.30 ± 0.02	3.16 ± 0.00	2.44 ± 0.02	1.98 ± 0.01	No
	100	4.48 ± 0.04	3.30 ± 0.03	2.56 ± 0.04	2.08 ± 0.02	No
1.0	50	4.62 ± 0.12	3.44 ± 0.01	2.71 ± 0.03	2.16 ± 0.08	1.82 ± 0.02
	100	4.98 ± 0.13	3.70 ± 0.02	2.90 ± 0.03	2.31 ± 0.08	1.92 ± 0.02
1.5	50	5.51 ± 0.05	4.10 ± 0.06	3.22 ± 0.02	2.61 ± 0.01	2.23 ± 0.05
	100	6.07 ± 0.06	4.51 ± 0.07	3.54 ± 0.03	2.85 ± 0.01	2.44 ± 0.06
2.0	50	6.27 ± 0.18	4.85 ± 0.21	3.72 ± 0.07	3.09 ± 0.22	2.41 ± 0.09
	100	7.03 ± 0.22	5.34 ± 0.14	4.16 ± 0.09	3.45 ± 0.25	2.68 ± 0.10
2.5	50	6.69 ± 0.07	4.96 ± 0.07	4.16 ± 0.12	3.32 ± 0.07	2.86 ± 0.06
	100	7.51 ± 0.05	5.60 ± 0.12	4.56 ± 0.12	3.71 ± 0.08	3.21 ± 0.11

No: we could not identify the migration time due to the overlap of several peaks.

Table 3
Resolution (R_s)[a] between the 50-bp and 100-bp DNA fragments according to the different electric fields and concentration of the PEO sieving gel by CGE–LIF ($n = 5$)

PEO (%)	Electric field (V/cm)				
	250	312.5	375	437.5	500
0.5	1.22 ± 1.22	0.35 ± 0.61	No	No	No
1.0	2.30 ± 0.18	2.13 ± 0.19	1.83 ± 0.20	1.76 ± 0.08	1.23 ± 0.11
1.5	3.91 ± 0.56	3.52 ± 0.35	3.17 ± 0.16	2.73 ± 0.14	2.49 ± 0.06
2.0	4.15 ± 0.46	3.96 ± 0.16	3.64 ± 0.27	2.99 ± 0.22	2.61 ± 0.43
2.5	6.38 ± 1.31	5.15 ± 0.19	4.93 ± 0.18	4.10 ± 0.15	3.50 ± 0.33

No: we could not calculate the resolution because of the overlap between two peaks.
[a] $R_s = 2(t_2 - t_1)/(w_1 + w_2)$, where t_1, t_2, w_1 and w_2 are the migration times and peaks widths of adjacent DNA peaks.

3.3. Application of clinical samples

In this study, the utility of CGE–LIF technology for the rapid detection of canine T-cell lymphoma (90-bp) was determined by comparing the PCR results of conventional gel electrophoresis with those from CGE–LIF. When the CGE–LIF method was applied to real clinical samples for the specific DNA fragment (90-bp DNA) of the TCRγ gene, the migration time and the corrected peak area showed relative standard deviations = 100 × S.D./mean) of 0.29% and 0.58% ($n = 5$), respectively (Table 4).

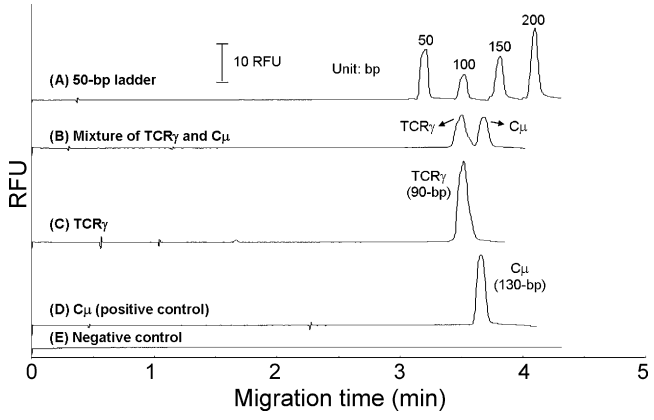

Fig. 2. Representative CGE–LIF electropherograms of TCR(and Cμ at the optimum CGE separation condition. CGE–LIF conditions: capillary, 50-μm I.D. fused-silica capillary; total length, 40 cm; effective length, 20 cm; excitation source, 5 mW He–Ne Laser (543 nm); (A) 50-bp DNA ladder, (B) mixture of TCRγ (90-bp) and Cμ (130-bp) (C) TCR(gene, (D) positive control C((130-bp) and (E) negative control; running buffer, 0.5 μg/mL EtBr in 1× TE buffer (pH 8.00); coating gel, 1.0% PVP (M_r 1,000,000); sieving matrix, 1.5% PEO (M_r 600 000) in running buffer; sample injection, electrokinetic injection at electric field of 75 V/cm for 30 s; sample separation electric field, 375 V/cm. RFU: relative fluorescence unit.

Fig. 3 shows the results of the CGE–LIF method for the investigations of PCR products from the nine canine blood samples. The clinical sample was analyzed within 4 min using the CGE–LIF method based on the condition with 1.5% PEO (M_r 600,000) and electric field strength of 375 V/cm. As per the clinical records, 4 of total 9 dogs were confirmed as having canine T-cell lymphoma using both conventional slab gel electrophore-

Table 4
Comparisons of the migration time, peak area and resolution (R_s)[a] of the TCR((90-bp DNA) and Cμ (130-bp DNA) fragments in the mixing sample at a sieving gel containing 1.5% PEO and an electric field of 375 V/cm by CGE–LIF ($n = 5$)

	Mixture of TCR(and Cμ	
	TCR((mean ± S.D.)	Cμ (mean ± S.D.)
Migration time (min)	3.43 ± 0.01	3.61 ± 0.01
Peak area	55.04 ± 0.32	44.47 ± 0.81
R_s	1.48 ± 0.06	

[a] $R_s = 2(t_2 - t_1)/(w_1 + w_2)$, where t_1, t_2, w_1 and w_2 are migration times and peaks widths of adjacent DNA peaks.

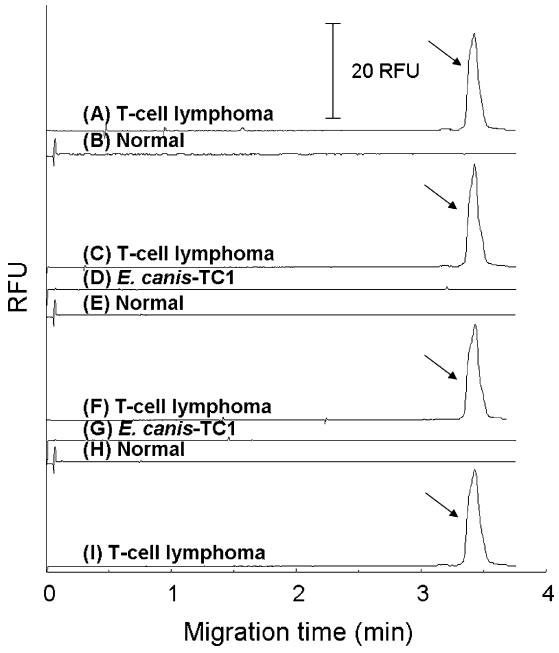

Fig. 3. CGE–LIF electropherograms for nine canine clinical samples used to detect T-cell lymphoma with TCRγ primers. The CGE–LIF conditions were the same as those shown in Fig. 2. Samples A, C, F and I were dogs infected with T-cell lymphoma. Samples B, E and H were normal dogs non-infected with T-cell lymphoma. Samples D and G were dogs infected with *E. canis*, but without rearrangement of TCRγ gene. Arrows indicate the TCRγ gene (90-bp DNA).

sis and CGE–LIF methods (Fig. 3A, C, F, I). According to a previous report [3], one dog with *E. canis* infection from 101 clinical samples showed a positive value for a diagnosis of T-cell lymphoma. However, in canine blood samples with our two *E. canis* infection, gene rearrangement for T-cell lymphoma was unrealized. These results were identified in conventional slab gel electrophoresis and CGE–LIF method that there was no amplified 90-bp DNA in our two *E. canis* infection samples (Fig. 3D and G). In supplementary investigations for total nine dogs, we could remind that the results of all samples were able to obtain within 4 min through the CGE–LIF method.

4. Conclusions

This study evaluated the utility of CGE–LIF analyses of the amplified PCR products for the rapid detection of canine TCR(gene disorder. After the PCR amplification of the specific TCR(gene in dogs, the amplified 90-bp DNA fragments were analyzed within 4 min by the CGE separation. When both slab gel electrophoresis and CGE–LIF were applied to nine real clinical samples for the specific DNA fragment of TCR(gene, both methods showed similar results. However, the differentiation of the specific TCR(gene (90-bp DNA) and positive control Cμ (130-bp DNA) in dogs could be accomplished with greater speed, simplicity and with smaller samples using CGE–LIF than with the conventional slab gel electrophoresis technology. This PCR/CGE–LIF technique is expected to be a new tool for the rapid diagnosis of the PCR-amplified DNA of canine T-cell lymphoma owing to its speed, small sample requirement and simplicity.

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