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Analysis of polymerase chain reaction-product by capillary electrophoresis with laser-induced fluorescence detection and its application to the diagnosis of medium-chain acyl-coenzyme A dehydrogenase deficiency

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Abstract

Capillary gel electrophoresis with laser-induced fluorescence (CGE–LIF) has been developed to detect polymerase chain reaction (PCR) amplified samples. LIF detection was performed using Thiazole Orange as the fluorescent intercalating dye. This method was ca. 100× as sensitive as that with UV detection. The highly sensitive CGE–LIF was applied to the detection of the most prevalent mutation (lysine^{329}-to-glutamic acid substitution) in medium-chain acyl-coenzyme A dehydrogenase (MCAD) deficiency. The disorder, which shows an autosomal recessive inheritance, is known to be highly prevalent among Caucasian population and often mimics as Reye-like syndrome or sudden infant death.

A DNA fragment containing the mutation site was PCR-amplified with two sets of allele specific oligonucleotide primers, followed by CGE–LIF. The mutant allele produced a 175-base pairs DNA fragment, which the normal allele generated a 202-base pairs DNA fragment. CGE–LIF clearly distinguished these PCR products, facilitating rapid diagnosis of MCAD deficiency.

1. Introduction

The polymerase chain reaction (PCR) procedure has been used to enzymatically amplify a specific segment of a target gene, which produces a 10^5-fold increase in the amount of target sequences. Detection of the PCR product is generally done by ethidium bromide staining after the separation by gel electrophoresis or hybridization with a radio-labeled DNA probe. These detection techniques are not suitable for routine clinical analysis, because of problems of rapidity, quantification, reproducibility and the repeated use of gel separation.

Recently, capillary electrophoresis (CE) has been used to analyze PCR products. For analysis of double-strand (ds) DNA fragments by CE, there are three methods which use low-, and zero-cross-linked polyacrylamide [1], agarose [2] and methylcellulose derivatives [3–6] as buffer additives for molecular sieving. We have studied capillary gel electrophoresis (CGE) with laser-induced fluorescence (LIF) detection for sensitive and direct analysis of PCR products. A

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low-cross-linked polyacrylamide gel (3% T, 0.5% C)[1], which was developed by Heiger et al. [1], was used as a CGE system with treated silica capillary. LIF detection was performed using Thiazole orange as the fluorescent intercalating dye. This method had higher sensitivity, and higher resolution than a CGE system with UV detection [7].

We have applied a highly sensitive CGE–LIF system to the detection of the most frequent mutation in medium-chain acyl-coenzyme A dehydrogenase (MCAD) deficiency. The deficiency, highly prevalent among Caucasians, is an autosomal recessive disorder that has been known to cause sudden infant death and Reye-like syndrome among children [8]. Most of the MCAD deficiency (90% of mutant alleles) are caused by a single nucleotide change (A to G) at nucleotide position 985, which substitutes glutamic acid for lysine at amino acid position 329. A DNA fragment containing the mutation site was PCR-amplified by two sets of allele specific oligonucleotide primers. The analysis of the PCR products by CGE–LIF system clearly distinguished between normal and mutant alleles, thus facilitating rapid diagnosis of MCAD deficiency.

2. Experimental

2.1. Apparatus

A P/ACE 2000 instrument with a LIF detector (Beckman, Fullerton, CA, USA) was used. Excitation was at 488 nm and a 530 nm band-pass filter was used for emission. A Model 270 A CE system (ABI, Foster City, CA, USA) was used. The separations were monitored on-column at 260 nm.

2.2. Materials

Hae III digested ΦX174 DNA was obtained from Nippon Gene (Osaka, Japan). Hinc II digested ΦX174 DNA and Hapa II digested pBR 322 were obtained from Toyobo (Tokyo, Japan). γ-Methacryloxypropyltrimethoxysilane was purchased from Sigma (St. Louis, MO, USA). Acrylamide, Bis, N,N,N′,N′-tetramethylethylenediamine (TEMED) and ammonium peroxydisulfate (APS) were from Wako (Osaka, Japan). Silica capillary tubing was obtained from GL sciences (Tokyo, Japan), and 37 cm (effective length 30 cm) × 100 μm I.D. × 375 μm O.D. for the P/ACE 2000 with a LIF detector, and 50 cm (effective length 30 cm) × 100 μm I.D. × 375 μm O.D. for the Model 270 A CE system were used.

AmpliTaq DNA polymerase was from Perkin-Elmer Cetus (Norwalk, CT, USA). Primers used for the PCR were synthesized on a Model 380A DNA synthesizer (ABI). Thiazole Orange was generously donated by Dr. Satow (Beckman Instruments, Tokyo, Japan). Other chemicals were reagent grade.

2.3. Procedures

CGE–LIF

Acrylamide polymerization was accomplished in the capillary according to the methods of Paulus and Ohms [9]. The capillary was rinsed with distilled water for 5 min.

A mixture of methanol–γ-methacryloxypropyltrimethoxysilane (50:50, v/v) was injected into the capillary and left there for 3 h. After washing with distilled water for 5 min, 5 ml of a solution consisting of acrylamide and Bis dissolved in 100 m*M* Tris–250 m*M* borate buffer (pH 7.8) was carefully degassed by vacuum and introduced into the capillary after adding both 2 μl of TEMED solution and 50 μl of 10% (w/v) APS solution. The polymerizing solution was quickly introduced into the treated capillary by a vacuum injection system equipped with a P/ACE 2000 for 5 min and left for at least 2 h. Sample injections were performed electrophoretically for typically 2 s at 3.7 kV.

Electrophoresis was performed with 100 m*M* Tris–100 m*M* borate buffer pH 8.3 containing 0.1 μg/ml thiazole orange and 2 m*M* EDTA at 7.4 kV, 30°C. The DNA fragments were detected

[1] C = g N,N′-methylenebisacrylamide (Bis)/%T; T = (g acrylamide + g Bis)/100 ml solution.

by LIF detection using the 488-nm line of an Ar ion laser for excitation, and 530 nm for emission.

Allele specific amplification of MCAD gene

DNA was extracted from dried blood specimens on Guthrie cards that are generally used for neonatal screening. Three discs (3 mm in diameter) containing about 10.8 μl of blood were punched from a specimen by a steel punch and extracted according to a previously described method [10].

Extracted DNA was subjected to PCR with two sets of primers. The first set consisted of sense primer M31 (5′-TATCATTTATGCTGGCTGAAATGGCAATCA-3′) and antisense primer M34 (5′-GGATATTCTGTATTAAATCCATTGCCTCCA-3′). It is capable of amplifying only the normal sequence, producing a 202-base pairs (bp) fragment, because the 3′-end of primer M31 is complementary to the normal MCAD sequence at nucleotide 985 (indicated by bold letter). To increase the specificity of amplification, a mismatched C residue (underlined) was introduced adjacent to its 3′-end. The second set of primers consisted of M32 (sense, 5′-TATCATTTATGCTGGCTGAAATGGCAATTG-3′) and primer M19 (antisense, 5′-CCAAGTATCTGCACAGCATC-3′). It is capable of amplifying only the mutant sequence, generating a 175-bp fragment, because the 3′-end of primer M32 is complementary to the mutant sequence at nucleotide 985 (indicated by bold letter). A mismatched T residue (underlined) was introduced adjacent to its 3′-end to increase the specificity of amplification. The principle of the allele specific amplification of MCAD gene is illustrated in Fig. 1. The reaction mixture contained 10 m*M* Tris–HCl (pH 9.0), 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.1% (w/v) gelatin, 1% Triton X-100, 0.2 m*M* of each dNTP (dATP, dGTP, dCTP and dTTP), 0.2 μ*M* of each primer, 2.5 units of Taq DNA polymerase and 10 μl of extracted DNA in a total volume of 100 μl. The two sets of PCR were performed separately for 30 cycles on a DNA Thermal Cycler (ATTO zymoreactor, Tokyo, Japan) under the following program: 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C. The two reaction mixtures were combined and analyzed by CGE–LIF.

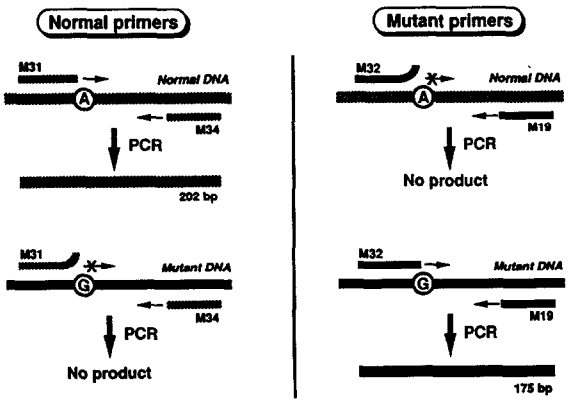
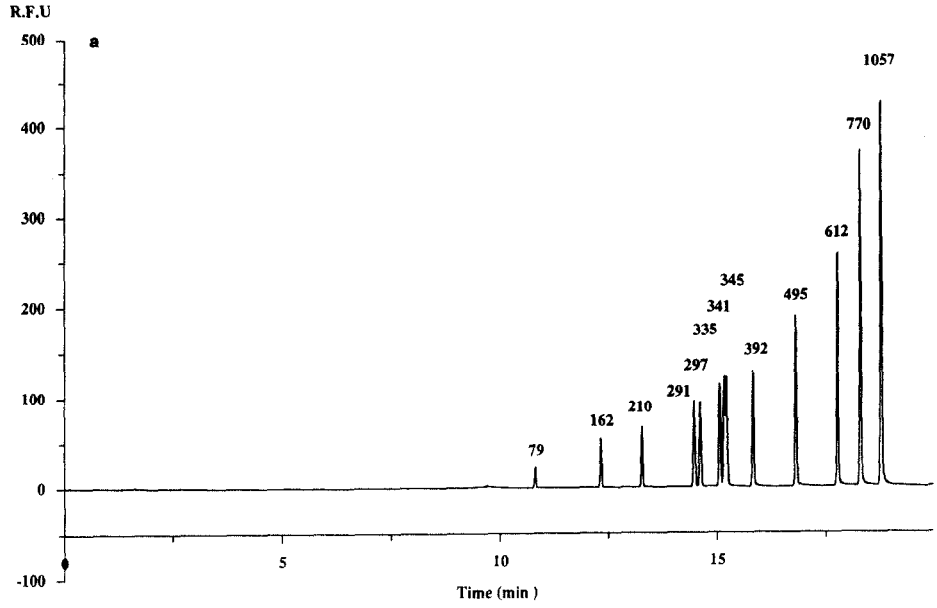


Fig. 1. Scheme of the method of allele specific amplification of the MCAD gene.

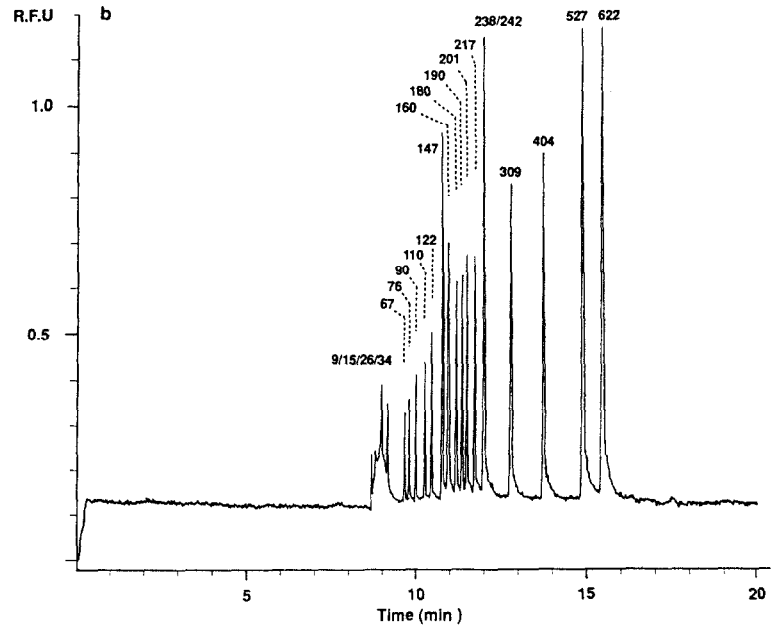
3. Results and Discussion

Schwartz and Ulfelder [7] recently developed a highly sensitive method of separating PCR fragments by CE–LIF which used hydroxypropylmethylcellulose as sieving buffer and thiazole orange as an intercalating dye. This method provides a useful technique to detect a specific gene from a small amount of human DNA after PCR, because it is ca. 100–400 times as sensitive as UV detection. However, the PCR product must be submitted to ultrafiltration to remove PCR reagent before analysis by CE–LIF, because PCR components co-migrate with the PCR product of interest in CE–LIF.

We directly analyzed PCR products without the purification of PCR product on CGE (3% T, 0.5% C) with LIF and the dye. Hinc II digested ΦX174 RF DNA and Hpa II digested pBR 322 were separated with at 3% T–0.5% C gel. Typical electropherograms obtained are shown in Fig. 2a and b. The electropherograms of ds DNA fragment obtained from Hinc II digested ΦX174 RF DNA show separation of the fragments differing by 4–6 base pairs (291/297, 335/341 and 341/345 bp) in length. The separation of Hpa II digested pBR 322 fragments were achieved due to excellent resolution obtained by CGE–LIF. The good resolution of ds DNA



Separation of ΦX174 RF DNA / HincII digest



Separation of pBR322 / Hpa II digest

Fig. 2. Separation of dsDNA fragment by CGE–LIF. (a) Separation of Hinc II digested ΦX174 RF DNA; (b) separation of Hpa II digested pBR 322. R.F.U. = Relative fluorescence unit.

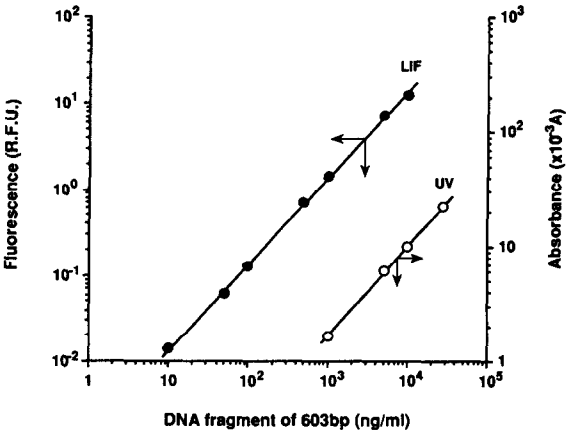


Fig. 3. A comparison of CGEs sensitivity obtained with LIF (●) detection and UV absorbance (○) detection at 260 nm.

restriction fragment by CGE–LIF may be obtained for the same reason that produced the effect of ethidium bromide (e.g., structure and charge-related effects), which was reported by Schwartz et al. [4] and Guttman and Cooke [11].

The sensitivity obtained by CGE–LIF was compared with that of CGE with UV detection. Samples of ΦX174 DNA/Hae III digest for standard curves were prepared as follows: the Hae III digested ΦX174 DNA was purified twice by ethanol precipitation and the obtained precipitate was diluted with 10 m*M* Tris–1 m*M* EDTA buffer (pH 8.0). The diluted ΦX174 DNA fragments were separated by CGE with LIF and UV detection. The peak height of 603 bp fragment obtained from LIF and UV detection were plotted against the concentration of 603-bp fragments. As shown in Fig. 3, the detection limits obtained from LIF and UV were ca. 10 ng/ml and 1000 ng/ml, respectively. CGE–LIF was ca. 100 times as sensitive as UV detection. The detection limit of DNA fragments by CGE–LIF was adequate to detect a specific gene amplified from a small amount of human DNA on dried blood spots after PCR.

The precision of the migration time obtained by this CGE–LIF for dsDNA fragments ranging from 72 to 1353 bp in Hae III digested ΦX174 DNA was examined. Within-run ($n = 10$) and day-to-day ($n = 6$) results are shown in Table 1. The mean within-run and day-to-day R.S.D.s were 0.55 and 0.71%, respectively.

The life of this gel-filled capillary was over 80 separations without appreciable decrease in performance.

3.1. Application of CGE–LIF analysis to diagnosis of MCAD deficiency

Allele specific amplification of the MCAD gene was performed to produce either a 202-bp DNA fragment, which indicates the presence of normal allele, or a 175-bp DNA fragment, which indicates the presence of mutant allele. Analytical results of the PCR products by CGE–LIF are shown in Fig. 4. DNA fragments amplified

Table 1
Precisions of migration time for various DNA fragment on the separation of Hae III digested ΦX174 RF DNA

No. of base pairs	Within-run ($n = 10$)		Day-to-day ($n = 6$)	
	Mean (min)	R.S.D. (%)	Mean (min)	R.S.D. (%)
72	11.39	0.34	11.19	0.57
118	12.52	0.48	12.16	0.61
194	14.25	0.62	13.67	0.49
234	15.01	0.65	14.35	0.39
271	15.77	0.66	15.00	0.74
281	15.98	0.64	15.21	0.75
310	16.48	0.67	15.64	0.73
603	19.70	0.58	18.64	0.78
872	20.60	0.42	19.61	0.85
1078	21.06	0.48	19.91	0.90
1353	21.42	0.52	20.20	1.03

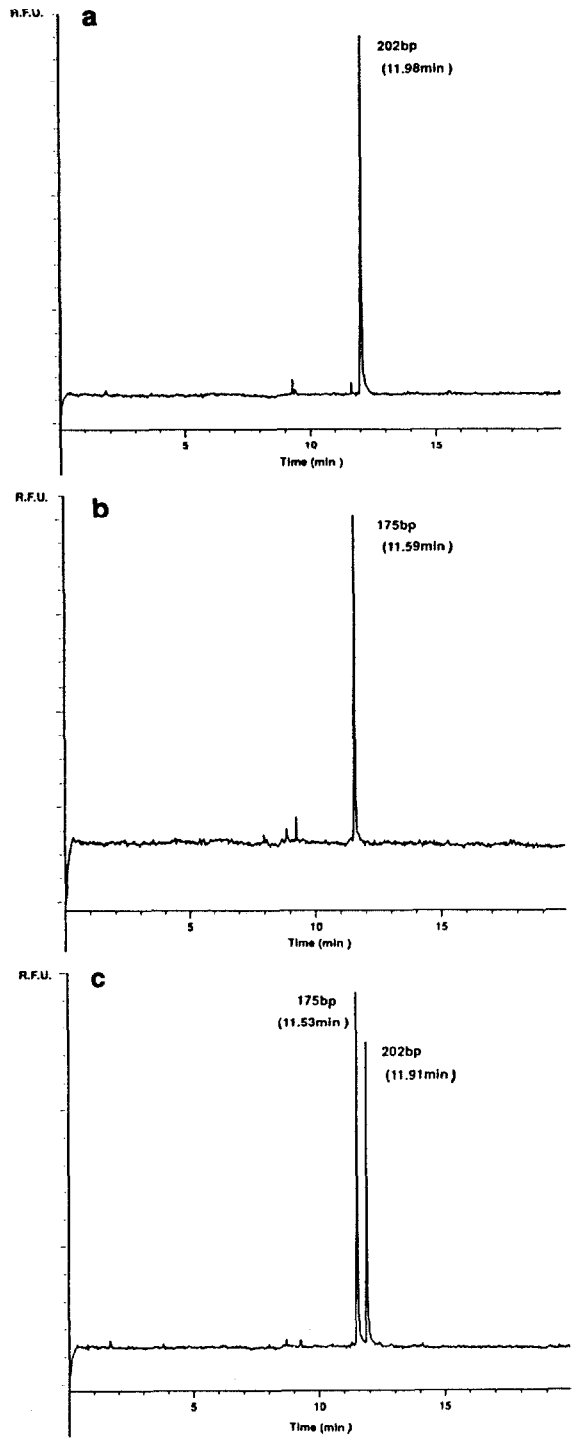


Fig. 4. Electropherograms of DNA fragments obtained by allele specific PCR. (a) Normal subject; (b) MCAD deficiency (homozygote); (c) heterozygous carrier of MCAD deficiency.

from normal and mutant alleles eluted from capillary gel after 11.5 min (175 bp) and 12 min (202 bp), respectively. A heterozygous sample shows two peaks of 175 bp and 202 bp (Fig. 4c), indicating that homozygotes, heterozygotes and normal subjects can be clearly distinguished by CGE–LIF within minutes. This clear diagnosis result stemmed from the high resolution of CGE–LIF: the extra peaks resulting from PCR mixture (dNTP and primers) were not present and the peaks of PCR products (175 bp and 202 bp) had good shapes. These results were better than those obtained with the sieving buffer reported by Schwartz and Ulfelder [7]. It is thought that an acrylamide gel capillary is more useful than hydroxypropylmethylcellulose for the analysis of relatively small DNA fragments (60–200 bp) obtained from PCR.

Various DNA diagnostic methods have been developed to detect the most prevalent mutation in MCAD deficiency [12]. These included modified PCR-NcoI digestion method, allele specific oligonucleotide hybridization method and allele specific PCR. Among these, the third, which is based on a method originally described by Newton et al. [13], appears to be most efficient. We [10,12] and others [14] have described allele specific PCR for MCAD deficiency, in which allele specific DNA products are separated by an agarose gel electrophoresis and detected by fluorescence or ethidium bromide staining. Application of CGE–LIF to the method has several advantages. First, it decreases the time required for the analysis without sacrificing resolution. Second, repeated use of the capillary gel is well tolerated with good precision as shown in our study. Third, high sensitivity obtained by CGE–LIF can reduce the amount of sample DNA and the amount of Taq DNA polymerase, which is a costly component of the methods utilizing PCR. Fourth, since the detection is readily amenable to automation, the method may be used in a clinical laboratory.

Presymptomatic diagnosis and appropriate dietary management are important in MCAD deficiency to prevent life-threatening complications. Our method described here would facilitate rapid diagnosis of this potentially fatal disorder.

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