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Journal of Chromatography A, 853 (1999) 519–525

JOURNAL OF
CHROMATOGRAPHY A

Short communication

High-throughput genotyping of factor V Leiden mutation by ultrathin-layer agarose gel electrophoresis

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Abstract

Ultrathin-layer agarose gel electrophoresis is a novel combination of the established methodologies of slab gel electrophoresis and capillary gel electrophoresis. This new format provides a multilane separation platform with rapid analysis time and excellent sensitivity by using laser-induced fluorescence scanning detection system. Sample injection onto the ultrathin-layer separation platform is easily accomplished by membrane mediated loading technology. In this paper, we demonstrate the sensitivity and high-throughput fashion of this novel separation and detection system for rapid genotyping of the coagulation factor V Leiden mutation by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis. The PCR amplified fragment from exon 10 of the factor V gene was digested by the Mnl I restriction enzyme, followed by automated ultrathin-layer agarose gel electrophoresis analysis with *"in migratio"* fluorescent labeling during the separation process. Due to its speed and automation, this method should be considered for large scale screening of factor V Leiden mutation. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Ultrathin-layer electrophoresis; Factor V Leiden mutation; DNA

1. Introduction

Inherited resistance to activated protein C (APC resistance) is one of the most common inherited risk factors associated with venous thrombosis (i.e., blood clotting that can cause thromboembolism or pulmonary embolism [1]). The prevalence of APC-resistance in the general population is 1–5% [2] and approximately 20–65% for patients with venous thromboembolism history [3,4]. Protein C (PC) is activated by the thrombin–thrombomodulin complex and by protein S cofactor on the endothelial cell surface (activated PC, or APC). In this anticoagulation cascade, APC selectively degrades coagulation factors Va and VIIIa [5]. The total inactivation mechanism of coagulation factor V (activated form) by APC includes several cleavage steps. Inactivation occurs mainly through cleavage at Arg(506) with a subsequent cleavage at Arg(306). Substitution of arginine by glutamine at position 506, as a consequence of a G→A substitution at position 1691 in exon 10 of factor V (FV) gene [6–8], produces an APC resistant FV molecule [9]. This type of resistant FV gene is called the factor V Leiden allele [10]. Since this variant lacks the critical Arg(506) APC cleavage site, inactivation of FV is largely impaired leading to excessive thrombin generation and a presumed life-long risk factor for thrombosis [11]. Leiden mutation may also be responsible for 40–60% of unexplained familial thrombophilia [12,13]. Since 80–94% of individuals positive to the APC-resistance test have Leiden mutation, this represent

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PII: S0021-9673(99)00573-7

one of the most common inherited risk factors associated with venous thrombosis [14,15]. This has prompted the development of high-throughput methods for rapid and accurate detection of carriers for factor V Leiden mutation.

Identification of point mutations is often based on the recognition of altered sequence by restriction endonucleases, producing different lengths of the digested DNA fragments (restriction fragment length polymorphism, RFLP) [16]. Identification of FV Leiden mutation by RFLP is based on the loss of a naturally occurring Mnl I restriction site at the exon 10 region of the FV gene [17]. Using PCR–RFLP as a genotyping technique, the proper region of the FV gene is amplified by polymerase chain reaction (PCR), followed by the Mnl I digestion of the PCR products. The resulting restriction fragments have different chain lengths, depending on the presence or the absence of the mutation. The most frequently used analysis methods of the digested PCR products are conventional agarose gel electrophoresis [18,19] and vertical polyacrylamide slab gel electrophoresis [20,21]; however, these methods require time consuming manual operation. Even the recently introduced capillary electrophoresis method in high-throughput/consecutive injection separation mode [22] requires 2.5 min/sample for the analysis.

In this paper, a rapid, sensitive and high-throughput screening method is described using automated ultrathin-layer agarose gel electrophoresis system with laser-induced fluorescence (LIF) detection for the analysis of the PCR–RFLP fragments. We compared the detection sensitivity and speed of manual polyacrylamide gel electrophoresis (PAGE) with the automated ultrathin-layer agarose gel electrophoresis technique and demonstrated the applicability of the later in high-throughput genotyping of FV Leiden mutation.

2. Materials and methods

2.1. Chemicals

Acrylamide, *N,N′*-methylene-bis-acrylamide, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), ammonium persulfate, urea, Tris, boric acid and $EDTA\text{-}Na_2$ were purchased from ICN Biomedicals (Costa Mesa, CA, USA). In the ultrathin gel platform, low electroendoosmosis (EEO) Amresco's Agarose-III (Solon, OH, USA) was used in 0.5× TBE buffer (45 m*M* Tris, 45 m*M* borate, 1 m*M* EDTA, pH 8.3). The 100 base pairs DNA (bp) ladder was from McFrugal (San Diego, CA, USA). All other electrophoresis grade chemicals were from Sigma (St. Louis, MO, USA). Deionized water (18 MΩ) was used for all buffer solutions and reactions (Millipore, Bedford, MA, USA).

2.2. PCR–RFLP

The 267 bp fragment of the FV gene, including position 1691 on exon 10, was amplified according to Bertina et al [23]. For the polymerase chain reaction an EasyCycler thermal cycler was used (EriComp, San Diego, CA, USA) with the forward primer (PR-6967) 5′-TGCCCAGTCTTAACAA-GACCA-3′ and the reverse primer (PR-990) 5′-TGTTATCACACTGGTGCTAA-3′ produced by IDT (Coralville, IA, USA). PCR was carried out in 50 μl reaction volumes, containing 200–500 ng genomic DNA, 10 m*M* Tris–HCl (pH 8.3), 1.5 m*M* $MgCl_2$, 200 μ*M* of each dNTP (Promega, Madison, WI, USA), 2 m*M* dimethyl sulfoxide (DMSO) (Sigma) and 1.5 U of Taq polymerase (Perkin-Elmer). Each reaction mixture was denatured at 95°C for 5 min followed by 36 cycles with 30 s denaturing at 95°C, 45 s annealing at 55°C, 45 s extension at 72°C, with a final 10 min extension at 72°C. Twenty microliters of each PCR reaction mixture were then digested with 1.9 U Mnl I enzyme (New England Biolabs, Beverly, MA, USA), 10 m*M* Tris–HCl, 10 m*M* $MgCl_2$, 1 m*M* dithiothreitol (pH 7.9 at 25°C) and 0.004 mg/ml bovine serum albumin (BSA) for 4 h or overnight at 37°C.

2.3. Separation and imaging methods

Polyacrylamide gel electrophoresis (PAGE) was carried out using a vertical electrophoresis system (Bio-Rad Labs., Hercules, CA, USA) with 8%T/5%C polyacrylamide gel[1] (16 cm×20 cm×0.1 cm)

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

by applying 10 μl volumes of digested PCR products and 4 μl of 100 bp standard ladder (Promega, Madison, WI, USA). Running conditions were 200 V applied voltage (22 mA) for 2.5 h using 0.5×TBE running buffer. When the separation process was accomplished, the electrophoresis gels were stained with 0.5 μg/ml ethidium bromide solution for 20 min and the image was captured using a Gel Doc 1000 system (Bio-Rad Labs.).

Automated ultrathin-layer electrophoresis: 2 g agarose powder was suspended in 100 ml 0.5×TBE buffer, boiled repetitively in a microwave oven until clear, and was kept at 60°C until use. A preheated (35–45°C) separation cassettes (18 cm×7.5 cm×190 μm) was filled with the melted agarose containing 50 n*M* ethidium bromide. After several minutes of cooling/solidification, the gel filled cassette was ready to be used. The DNA fragments were injected by previously discussed membrane mediated loading technology [24]. The injection end of the ultrathin separation slab gel had a straight edge with no individual wells. Small amounts of samples (0.2–0.5 μl) were spotted onto the tips of the 32 tab membrane loader (Genetic BioSystems, San Diego, CA, USA) and carefully inserted in close proximity to the straight edge of the separation gel. After the insertion of the spotted loading membrane, the electric field was applied and the DNA fragments were quantitatively loaded into the separation matrix from the membrane. The DNA molecules were fluorescently visualized during the separation process by *"in migratio"* intercalation with ethidium bromide that was dissolved in the separation gel-buffer. The labeled DNA fragments were imaged by the scanning laser-induced fluorescent detector of the system during their migration in real time [25,26]. The used gels were simply replaced by pumping fresh melted agarose into the separation cassette after each run. The effective separation length of the ultrathin-layer agarose gel was 6 cm. The applied separation voltage was 750 V, generating 5–7 mA of current.

3. Results and discussion

The principle of PCR–RFLP based analysis of the factor V Leiden mutation is shown in Fig. 1. First the 267 bp fragment from exon 10 of the FV gene was amplified by PCR. This section includes the possible Leiden mutation site (position 1691). Then the PCR product is digested with Mnl I restriction enzyme which cuts specifically at $5' \ldots CCTC(N)_7 - \ldots 3'$/$3' \ldots GGAG(N)_6 - \ldots 5'$. The amplified 267 bp fragment normally contains two sections of this particular recognition sequence, 163 bp apart from each other (indicated by the two boxes in Fig. 1A). In a normal case when no mutation is present, three fragments are produced with lengths of 67 bp, 163 bp and 37 bp as a result of the digestion (Fig. 1A). The Leiden mutation alters the Mnl I cleavage site at position 1691, thus the enzyme does not recognize it as a digestion site. Therefore, when the mutation is present, the PCR product will have only one recognition site for Mnl I, resulting two fragments with 67 bp and a 200 bp lengths (Fig. 1B). The existence of this one remaining restriction site serves as an internal control of the restriction digestion in the analysis of the mutant gene.

After the PCR–RFLP process is finished, the resulting DNA fragments are analyzed by gel electrophoresis and the fragment sizes determined. In this paper we compare the conventional polyacrylamide slab gel technique with a novel automated ultrathin-layer agarose gel electrophoresis method. Fig. 2 exhibits the separation of the PCR–RFLP fragments of 18 patient samples (lanes 1–18; lane S: 100 bp ladder sizing standard) using conventional polyacrylamide slab gel electrophoresis with post separation ethidium bromide staining. The injected amount was 10 μl PCR–RFLP product per lane (corresponds to approximately 0.2 μg DNA). As one can see, the 163 bp and 200 bp fragments were well separated, the 67 bp fragment was also detected. The 37 bp fragment could not be seen, probably because it co-migrates with the Bromophenol Blue tracking dye. Fig. 2 clearly shows, that patient samples 1, 2, 4–7, 9–17 exhibit only the 163 bp fragment in addition to the control 67 bp fragment; therefore, they are considered to be homozygous normal for the Leiden mutation. Patients 3, 8 and 18 exhibited both the 163 bp (refers to normal) and the 200 bp (refers to mutant) fragments; therefore, they are considered to be heterozygous for the mutation.

The very same PCR–RFLP products were also analyzed by automated ultrathin-layer agarose gel

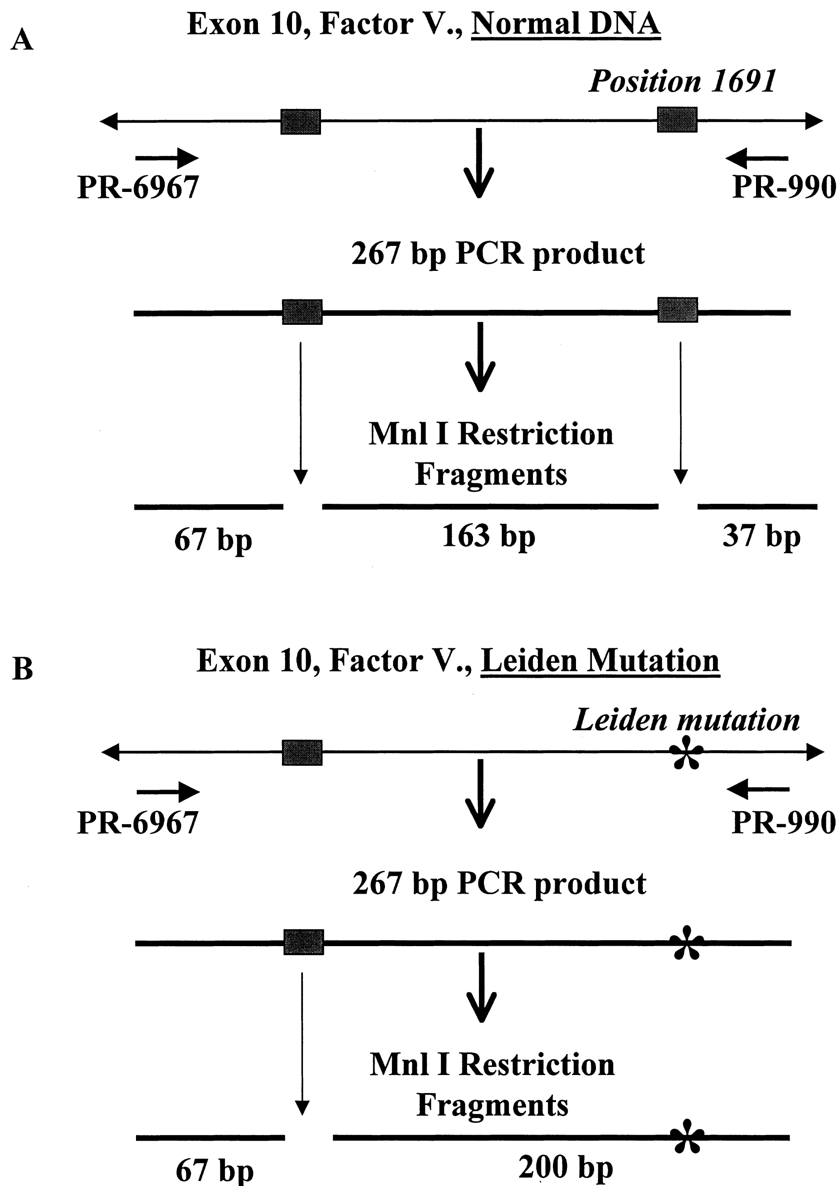

Fig. 1. Schematic diagram of PCR–RFLP analysis of the factor V Leiden mutation. PCR amplification of the FV gene region from genomic DNA was made as a first step, using PR 6967 and PR 900 primers. The PCR product (had two cleavage sites on the normal DNA in this region, indicated by the two boxes on A) was digested with Mnl I restriction enzyme, resulting in three restriction fragments (67 bp, 200 bp and 37 bp). The Leiden mutation alters the Mnl I cleavage site at position 1691, making it unrecognizable for the enzyme and resulting in only two fragments with 67 bp and a 200 bp lengths (B).

electrophoresis, injecting only 0.5 μl samples (corresponds to approximately 2.5 ng/lane) in the same order as above. As Fig. 3 depicts, very high sensitivity separation of all the PCR–RFLP fragments were obtained with excellent resolution, including the 37 bp fragment that can be clearly identified here. The

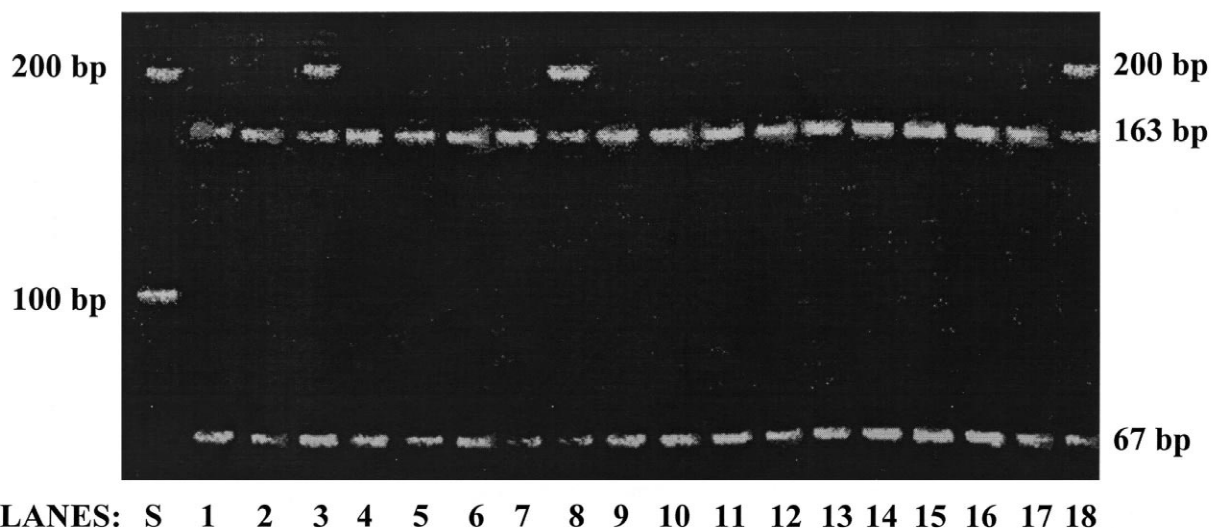

Fig. 2. Factor V Leiden genotyping of 18 patients by conventional polyacrylamide slab gel electrophoresis. Conditions: separation was performed by a manual, vertical polyacrylamide gel electrophoresis system, using 8%T/5% C polyacrylamide gel in 0.5×TBE running buffer, pH 8.3. Applied voltage: 200 V (22 mA) for 2.5 h. After the separation, the polyacrylamide gel was stained with ethidium bromide (0.5 μg/ml) for 20 min. Ten μl PCR–RFLP products were loaded on each lane (1–18) and 4 μl of 100 bp standard (lane S).

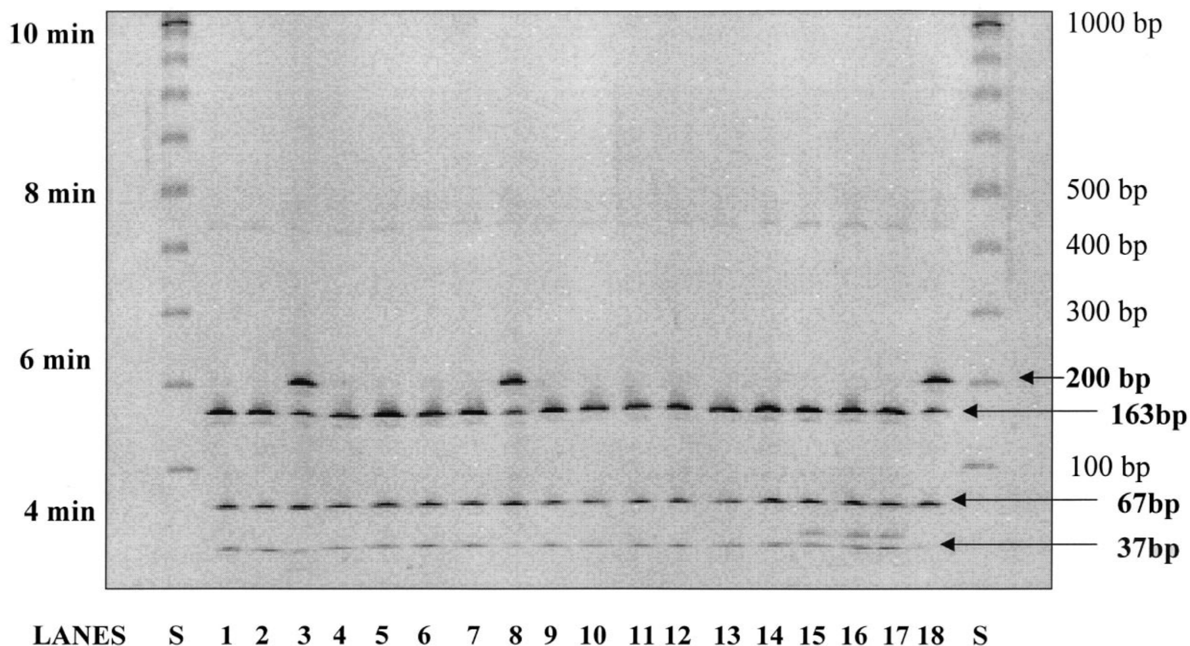

Fig. 3. Factor V Leiden genotyping of 18 patients by automated ultrathin agarose layer gel electrophoresis. Conditions: 2% agarose gel in 0.5 × TBE buffer containing 50 n*M* ethidium bromide. Running buffer: 0.5 × TBE; voltage: 750 V (7 mA); injection: membrane mediated, 0.5 μl/tab (samples were loaded in the same order as in Fig. 2).

extra bands above the 37 bp products in lanes 15–17 probably represent aspecific products. One can also observe that in lanes 3, 8 and 18, the 37 bp fragment band is weaker compared to the same bands in other sample lanes. This phenomenon is probably due to the reduced amount of this particular fragment in the heterozygous digestion mixture. The figure also shows that real-time fluorescent detection makes possible the identification of all the PCR–RFLP products in less than 6 min for up to 20 lanes (18 samples plus 2 standard ladder lanes: S) that corresponds to 0.3 min per sample in high-throughput operation. Please note, that this method is 30 times faster than PAGE and almost 10 times faster that of the recently introduced capillary electrophoresis using multiple-injection mode (separation time: 2.5 min per sample).

4. Conclusions

Genotyping of factor V Leiden mutation by PCR–RFLP– automated ultrathin-layer agarose gel electrophoresis showed very consistent results that of obtained by conventional, horizontal polyacrylamide slab gel electrophoresis. Analyzing the same samples with both methods, patients 1, 2, 4–7, 9–17 were found to be normal, while patients 3, 8 and 18 were found to be heterozygous carrying factor V Leiden mutation. The extremely sensitive scanning LIF-based detection enabled injection of 80 times smaller amount of DNA onto the ultrathin-layer separation format. In addition, the required amount of ethidium bromide in the gel-buffer system was significantly lower (50 n*M*). Also, genotyping with automated ultrathin-layer agarose gel electrophoresis proved to be considerably faster in a per-sample basis (0.3 min/sample) compared to 9 min per sample time requirement of the conventional PAGE and 2.5 min/sample of the CE method. Our results clearly demonstrate that rapid genotyping of factor V Leiden mutation by PCR–RFLP fragment analysis can be accomplished with automated ultrathin-layer agarose gel electrophoresis in a very sensitive and high-throughput manner.

Acknowledgements

The authors gratefully acknowledge Dr. Tamas Toth (H-Med, Budapest, Hungary) for his stimulating discussions. This work was supported by the US–Hungarian Science and Technology Joint Research Fund, Project JF 654/96 and OTKA T022608.

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