

Double Fluorescent-amplification Refractory Mutation Detection (dF-ARMS) of the Factor V Leiden and Prothrombin Mutations

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Summary

Simultaneous fluorescent [F] detection of the factor V Leiden (G1691A) and the prothrombin 3'-untranslated region (G20210A) mutations were performed in a single tube polymerase chain reaction (PCR). Amplification refractory mutation detection system (ARMS) formed the basis of this assay design. Fluorescent-labelled primers incorporated into amplicons during the reaction facilitated detection directly by GeneScan analysis without further manipulation. To test the efficacy of this double [F]-ARMS (dF-ARMS) method, 48 patients with unexplained thrombotic tendencies were investigated for their factor V Leiden and prothrombin genotypes. These results corresponded exactly with data achieved using the more conventional methods of restriction fragment length polymorphism (RFLP)-PCR and direct DNA sequencing. Three out of the 48 patients in this group were found to be compound heterozygotes.

Introduction

Blood coagulation is a complex cascade of reactions between platelets, plasma factors and the vascular endothelium. These events culminate in massive thrombin formation which leads to the formation of an insoluble fibrin clot. Feedback mechanisms together with other control systems ensure that inappropriate or excessive activity of the participating enzymes is avoided.

Venous thrombosis is a serious health problem with an incidence of 1 per 1000 annually. Several genetic and acquired risk factors are known (e. g. surgery, pregnancy, oral contraceptives among others). Mutations in genes encoding the blood clotting pathway components play an important role in the predisposition to venous thrombosis. Variant alleles were previously defined for genes encoding protein C, protein S, antithrombin III and fibrinogen. However, these were recognised in fewer than 5-10% of thrombotic patients (1, 2).

In 1993, activated protein C resistance (APCr) was described (3) and this is now regarded as the most prevalent coagulation abnormality associated with venous thrombosis (2-5). It occurs in an estimated 20-65% of patients with a history of familial venous thromboembolism (5, 6). Genomic analysis clearly demonstrated that a G to A transition at nucleotide position 1691 in the factor V gene was responsible for 90-95% of APCr cases (7-9). This mutation produces a factor Va molecule, factor V Leiden, with an arginine (R) to glutamine (Q) amino acid substitution at position 506 in the polypeptide, rendering the protein partially resistant to inactivation by APC (5, 7). The prevalence of the mutation in the general population among Western countries varies between 2 and 15% (1). Recent data, however, suggests that this may be influenced by geographic location and ethnic background (10).

A genetic variant in the prothrombin gene (factor II) was described in 1996 by Poort et al. (11) resulting in a G to A transition mutation at nucleotide position 20210 in the 3'-untranslated region (UTR) of this gene. This mutation increases the concentration of prothrombin in plasma which in turn is associated with an increased risk of thrombosis (12-15). The prevalence of the mutation in the prothrombin 3'-UTR is estimated at about 2% in the healthy population and 6% in unselected consecutive patients with deep vein thrombosis (12, 16).

APCr can be detected by a variety of coagulation-based assays (14, 17). However these vary in specificity and sensitivity and do not permit accurate evaluation of patients on anticoagulation therapy or those possessing lupus anticoagulants (5, 18). For definitive diagnosis of the factor V Leiden and prothrombin mutations, an accurate DNA-based assay would be preferable. Some of the more recent strategies developed were based on allele-specific amplification (18, 19), whilst others required post-amplification procedures such as restriction enzyme analysis using MnlI and HindIII (7, 20-24) to identify these point mutations.

This report details the development of a double [F]-ARMS PCR assay which facilitates direct diagnosis using high resolution separation techniques based on GeneScan technologies. Fluorescent-labelled oligonucleotide primers are incorporated into the PCR reaction mixture and the resulting amplicons are visualised by fluorescence spectroscopy in an ABI Prism™ 310 analyser. The approach described here requires a single tube amplification reaction followed by direct GeneScan analysis. Furthermore dF-ARMS can be performed on whole blood and/or purified genomic DNA as an amplification template.

Materials and Methods

Blood Samples and DNA Preparation

Sodium citrate blood samples were collected from 48 consecutive patients attending anticoagulation monitoring clinics. The patients were receiving anticoagulant therapy because of a previous thrombotic episode associated with normal proteins C, S and antithrombin III. The white cell content from each blood sample was separated by centrifugation for 10 min at 2000 × g at room emperature and the buffy coat was removed prior to DNA extraction. Total

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Table 1 Oligonucleotide primers for detection of the factor V Leiden and prothrombin mutations by dF-ARMS

Primer	Type	Sequence	Length	Localization	Label	Size(bp)	Reference
FV-C	sense	5'-CTT TCA GGC AGG AAC AAC ACC-3'	21	Ex 10	u.l.	-	(25)
FV-N	antisense	5'-GGA CAA AAT ACC TGT ATT GCT **C**-3'	22	Ex 10/Intr 10	FAM	230	(25)
FV-M	antisense	5'-ATG GAC AAA ATA CCT GTA TAC CTT-3'	24	Ex 10/Intr 10	HEX	233	(25)
FII-C	sense	5'-TCT AGA AAC AGT TGC CTG GCA-3'	21	Intr M	u.l.	-	This study
FII-N	antisense	5'-CAC TGG GAG CAT TGA GGG TC-3'	20	poly A signal	FAM	340	This study
FII-M	antisense	5'-CAC TGG GAG CAT TGA GGC **A**T-3'	20	poly A signal	HEX	340	This study

Notes:

C= common, N= normal and M= mutant; Intr= intron, Ex= exon; FAM= 6-carboxyfluorescein, HEX= hexachloro-6-fluorescein; u.l.= unlabeled.

The underlined bases in each case represent the mismatch necessary for dF-ARMS and the bolded 3'-nucleotide in both N and M primers signify the allelic site.

Primers designed to amplify the normal and Leiden factor V alleles were taken from previously published work (25 and Genbank accession number M16967). The forward primer (FV-C) corresponded to nucleotides 1475-1500, the reverse primer (FV-N) to position 1701-1691 plus 11 nucleotides into intron 10 and the reverse primer (FV-M) to position 1701-1691 plus 13 nucleotides into intron 10. Those used to detect the prothrombin mutation were based on the human prothrombin intron M sequence for the forward primer corresponding to the nucleotides 26463-26483 and the 3'-end of the poly A signal for the antisense primers corresponding to the nucleotides 26803-26784 (31 and Genbank accession number M17262). Thus the mutant primer (FII-M) differs from the prothrombin-normal (FII-N) at the 3'-position. The penultimate mismatch nucleotides were chosen according to the empirical suggestions made by Newton et al. (32).

genomic DNA was recovered from these white cells using a QIAamp Blood Kit (Qiagen Ltd., West Sussex, UK), according to the manufacturer's instructions. DNA was quantified by measuring the absorbance at 260 nm.

Alternatively, whole blood can be used as a starting template, and is processed as follows. One ml of whole blood is heated to 95° C for 5 min and cooled to 30° C for 30 s. This step is repeated three times, after which the PCR reaction mixture (as outlined below) is added. Amplification conditions are outlined below.

Oligonucleotide Primers

Each sample was investigated using dye-labelled primers, directed at both the factor V and prothrombin genes. Genotypes were determined in a single tube PCR reaction consisting of a common forward primer which was unlabelled, together with two antisense primers, one of which detected the normal allele and the second detected the mutated allele. These antisense primers were differentially labelled. All primer characteristics together with other relevant details are given in Table 1. Oligonucleotide primers for the dF-ARMS protocol were synthesised and purified by Applied Biosystems (Applied Biosystems, Warrington, UK).

dF-ARMS Detection of Factor V Leiden and Prothrombin Mutations

Amplification reactions were performed in a final volume of 50 µl consisting of 500 ng genomic DNA template, 1X PCR buffer [54 mM Tris-HCl, pH 8.8, 5.4 mM $MgCl_2$, 5.4 µM EDTA, 13.3 mM $(NH_4)_2SO_4$], 300 µM of each dNTP, 8% DMSO, 8 mM β-mercaptoethanol, 0.4 mg/ml BSA, 15 pmoles of each primer (Table 1) and 2 U of Taq DNA polymerase (Sigma, Poole, UK). The reaction mixture was overlaid with mineral oil and amplification was carried out in a Pharmacia LKB Gene ATAQ Controller (Pharmacia, Uppsala, Sweden), using the following amplification profile: initial 5 min denaturation at 94° C, followed by 30 cycles consisting of 1 min denaturation at 94° C, 1 min annealing at 62° C and 1 min extension at 72° C. Samples were maintained at 72° C for 7 min before holding at 4° C prior to analysis.

GeneScan Analysis

For GeneScan analysis 0.5 µl of GeneScan™ 500 TAMRA (N,N,N',N'-tetramethyl-6-carboxyfluorescine) internal size standard (Applied Biosystems, Warrington, UK) was mixed with 1.0 µl of the PCR reaction and 12 µl HPLC water. Electrophoresis was performed through a 2.5% GeneScan polymer in 1X GeneScan buffer using a 46 cm capillary, according to the manufacturers recommendations. Fluorophore excited emissions were detected in an ABI Prism™ 310 analyser, using virtual filter C. All data peaks were automatically sized by comparison with the internal lane standard above. A dedicated Macintosh "PowerMac" computer (Apple Computer Inc., Foster City, CA) was used to store data.

Results

A dF-ARMS protocol was developed to genotype the factor V Leiden and prothrombin mutations in a single-tube reaction. Two sets of three primers were included in each reaction mixture and were used to detect normal and abnormal alleles simultaneously. Each primer set consisted of an unlabelled forward primer together with two differentially labelled reverse primers. Additional mismatches were included towards the 3'-ends of the reverse primers in order to increase the specificity of the reaction (Table 1). Primers directed at the factor V gene were taken from previously published work (25), and those for the prothrombin gene were specifically designed for this study. Exon 10 in the factor V gene and the 3'-UTR region of the prothrombin gene were amplified from normal individuals together with both homozygotes and heterozygotes for the R506Q and the G20210A mutations. Inclusion of dye-labelled primers permitted the generation of [F]-labelled amplicons during PCR. These products can be detected directly (without post-amplification modification), by genescanning (in an ABI Prism™ 310 analyser or similar instrument). Several annealing temperatures ranging from 58 to 68° C were tested in order to optimise reaction conditions. Clear discrimination of all genotypes was achieved at an annealing temperature of 62° C. Fig. 1 (a-e) shows the expected results of the dF-ARMS assay.

When a normal individual was analysed two 6-carboxyfluorescine (FAM)-labelled peaks (blue) were detected corresponding to the normal allelic sizes of 230 and 340 bp for the factor V and prothrombin genes respectively (Fig. 1a). An additional hexachloro-6-fluorescine (HEX)-labelled peak (black) was detected whenever the abnormal allele was present. Fig. 1b displays an individual with a normal prothrombin gene (blue peak only of molecular weight 340 bp) and a heterozygotic factor V locus (black and blue peaks). The reverse situation is shown for the individual analysed in Fig.1c. A subject who was homozygous (abnormal) for either allele, displays a single HEX-labelled peak at the corresponding position (Fig. 1d). Finally a compound heterozygote was identified in Fig. 1e showing HEX-labelled peaks at both loci. Amplified DNA fragments were also analysed by restriction fragment length polymorphism (RFLP)-PCR and direct DNA sequenc-

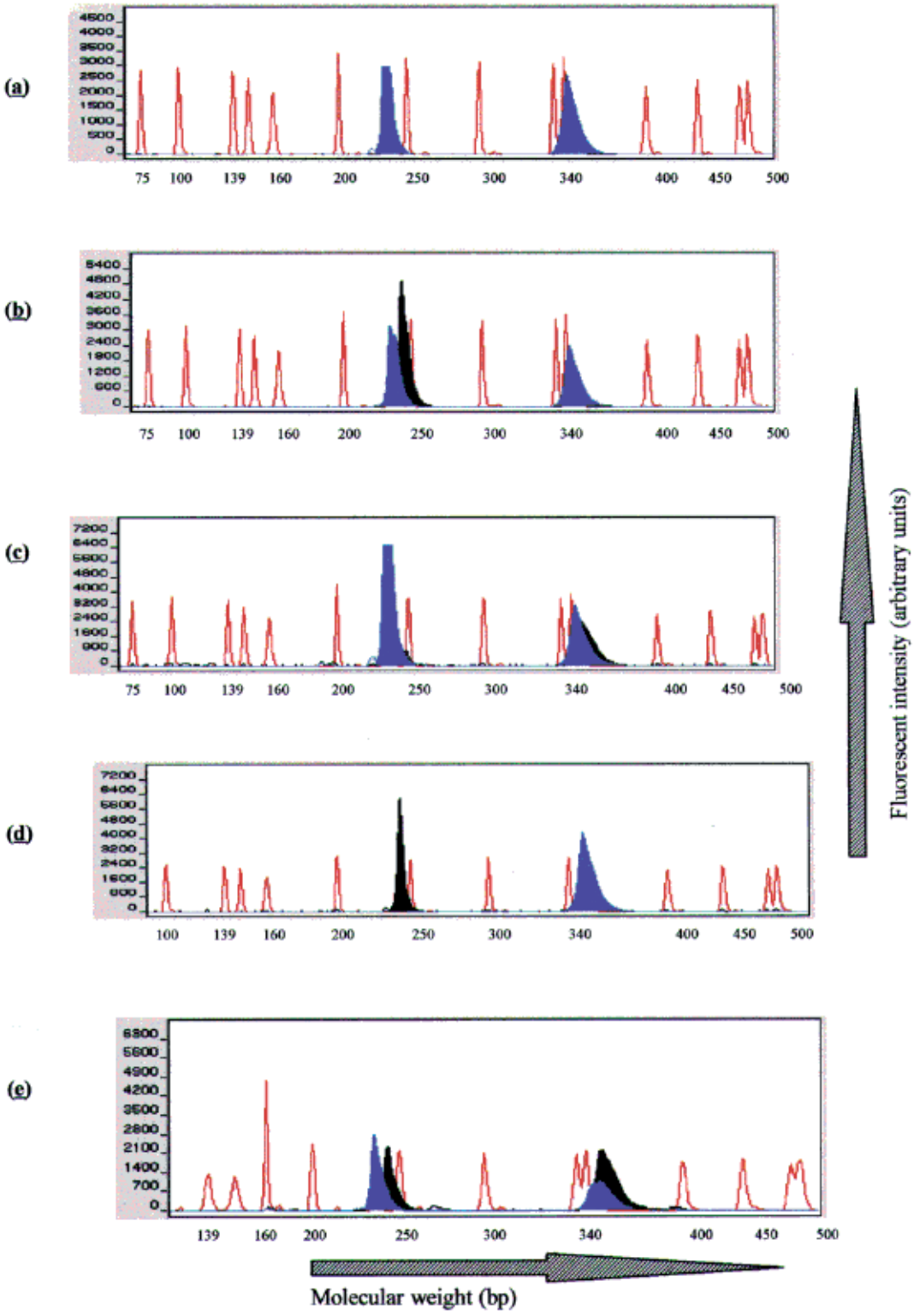

Fig. 1 Detection of factor V Leiden and prothrombin alleles using dF-ARMS. Blue peaks represent 6-FAM-labelled amplicons derived from the normal factor V and prothrombin genes. Black peaks correspond to HEX-labelled abnormal mutant amplicons (Table 1). All peaks were sized directly by comparison with the TAMRA-labelled internal standard (red peaks). Corresponding peak sizes are indicated in Table 1. Genotypes are given as follows: (a) Normal control, (b) factor V Leiden heterozygote and prothrombin normal allele, (c) factor V normal allele and prothrombin heterozygous control, (d) factor V Leiden homozygote and prothrombin normal allele, (e) compound heterozygote (factor V Leiden and abnormal 3'-UTR prothrombin alleles)

ing (data not shown). Both methods confirmed the validity of the dF-ARMS approach.

In order to test the efficacy of dF-ARMS, 48 consecutive patients (with an unexplained history of thrombosis but with normal proteins C, S and antithrombin III), attending an anticoagulation monitoring clinic, were referred to the Molecular Diagnostics Unit at CIT for genotyping. After genomic DNA extraction all samples were subjected to dF-ARMS. Briefly 44% (21/48) of this patient group were heterozygous at the factor V locus compared to only 10% (5/48) carrying the G20210A mutation in the 3'-UTR of the prothrombin gene. Three out of the 48 patients (6%) were found to be compound heterozygotes. These results were also confirmed by RFLP-PCR as outlined previously.

Discussion

Thrombophilia is now recognised as a multigenic disease (12). Comparison of data obtained from thromboelastography (TEG) measuring the real-time visco-elastic clotting properties of an individual's blood, and genotype analysis of factor V and prothrombin clearly demonstrates the necessity for a multiplex-based molecular approach to investigate this complex disease (Maher et al., submitted). Factor V Leiden and the more recently described prothrombin 3'-UTR mutations increase the risk of thromboembolic disease. Much remains to be uncovered concerning the importance of these and other mutations [e. g. methylenetetrahydrofolate reductase (MTHFR) C677T mutation (26, 27) leading to hyperhomocysteinaemia], and how they relate to other conditions on a broader basis (28). Factor V Leiden is known to interact with acquired factors (e.g. oral contraceptives, surgery, cancer and others), whilst the prothrombin mutation has been identified in 18% of selected patients with familial thrombophilia (11). Furthermore, both mutations are now thought to be associated with coronary artery disease, as indicated by a study on a selected group of young women presenting with myocardial infarction (12).

Several novel PCR strategies have been described to detect the latter mutations individually (7, 11, 18-25). The rate-limiting step in many of these protocols is DNA isolation. Genotyping using both whole blood and purified genomic DNA templates have been previously reported. Gómez et al. (21) used a small volume of whole blood in a multiplex-PCR approach to amplify the regions of interest from both the factor V and prothrombin genes. Allelic determination followed by simultaneous restriction enzyme analysis and careful interpretation of the agarose gel was required to ensure the assignment of correct genotype(s). Ripoll et al. (20) combined detection of both mutations using purified DNA and Hézard et al. (19), described an allele specific amplification (ASA)-PCR method requiring two reaction mixtures (normal and abnormal) for each template with either purified DNA or whole blood. In yet another novel approach, Bowen et al. (27) used a triplex-PCR design, followed by post-amplification heteroduplex analysis, to genotype factor V, prothrombin and MTHFR alleles. Clearly allele-specific PCR can be used to directly assess the genotype of significant alleles and is amenable to large-scale screening (28).

After DNA preparation either by purification or using a small volume of whole blood, the dF-ARMS-PCR method described in this paper facilitates the rapid and simple evaluation of an individual's gene profile. Essentially this method depends on the correct selection of a complementary allele-specific primer during the PCR reaction, producing the corresponding dye-labelled amplicon. Presence or absence of a coloured peak in the GeneScan data is sufficient to permit direct and unambiguous allelic determination (29). Problems concerning the interpretation of complex gel patterns are frequently encountered due to inadequate DNA fragment separation and visualisation. These difficulties can be overcome using high-resolution separation techniques, such as those based on GeneScan technologies (28, 29). No additional post-amplification procedures (such as restriction enzyme digestion or DNA sequencing) are necessary.

The efficacy of dF-ARMS was tested on 48 patients attending an anticoagulation clinic and from whom a sample of blood was taken and referred to CIT for factor V Leiden and prothrombin genotyping. Three out of the 48 patients (6%) were found to be compound heterozygotes. When compared to more conventional methods of analysis such as restriction fragment length polymorphism (RFLP)-PCR, these results confirm the validity of the dF-ARMS approach.

Finally for multigenic diseases like thrombophilia, multiplex-allele specific amplification methods will play an increasing role in defining an individual's genotype.

Unambiguous data output will facilitate the rapid implementation of effective therapeutic measures (30). Ultimately these methods will contribute towards reducing health economic costs.

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