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SIMULTANEOUS DETECTION OF FVL AND MTHFR (C677T) MUTATIONS USING PCR-RFLP METHOD FOR RAPID SCREENING OF INDIAN POPULATIONS

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ABSTRACT

Factor V Leiden (G1691A) and MTHFR (C677T) mutations are the two predisposing genetic polymorphisms for thromboembolism and so have been associated with a number of disorders like coronary artery disease, neural tube defects, pregnancy complications, Down 's syndrome, in vitro fertilization failure etc. The present attempt was made to develop a multiplex PCR RFLP method for diagnosis of FVL and MTHFR (C677T) mutations using previously published protocols but with half the operational time, which may be used as a cost and time effective diagnostic tool for rapid screening of Indian populations, devoid of prothrombin mutations.

Key Words: *Multiplex PCR, FVL, MTHFR*

INTRODUCTION

Factor V Leiden (G1691A) and MTHFR C677T mutations are the two predisposing genetic polymorphisms for thromboembolism and so have been associated with a number of disorders like Coronary Artery Disease (Frosst *et al.*, 1995, Sinha *et al.*, 2010), neural tube defects (Wilcken *et al.*, 1997), pregnancy complications (Mukhopadhyay *et al.*, 2009), down's syndrome (Cyrus *et al.*, 2009), In Vitro Fertilization failure (Hussein *et al.*, 2006) etc. Coinheritance of these two mutations increases the relative risk of thrombosis which is the basis of many pathological conditions.

FVL (G 1691 A) substitution, located on chromosome 1q23, results in a form of factor Va that is resistant to Activated Protein C (APC) leading to a relatively hypercoagulable state and thus disturbing the coagulation cascade. The metabolic pathway catalyzed by MTHFR enzyme is drastically disturbed resulting in hyperhomocysteinemia, when there occurs a substitution at 677 nucleotide in exon 1 of MTHFR gene located on 1p 36.3. These biochemical imbalances result in the rapid increase in the magnitude of various diseases, thus to control the mortality and morbidity caused by these substitutions, conducive environment is required like nutritional supplementation (Folic acid and Vitamin B12), Anti Thrombotic Agents (Aspirin & Low Molecular Weight Heparin). To employ these therapeutic measures, counseling and genotypic profiling of these mutations is essential.

In conjunction with these two variants, Prothrombin (G20210A) has also been found to be an independent risk factor for thrombosis and has been used for genotypic profiling in European countries; but in Indian clinical studies it has been found to be monomorphic/ infrequent (Kumar et al 2005, Sharma *et al.*, 2006). The author also screened four hundred subjects of North Indian population for the same variant revealing similar results, thereby this polymorphism might be unlikely to be involved in genetic testing and counselling in such populations.

For the simultaneous detection of these three markers with high throughput and in confined period of time, several techniques have been developed in different countries including RT-PCR (Von ashen *et al.*, 1999), Fluorescence scanning (Saffroy *et al.*, 2002), MS-PCR (Bowen *et al.*, 1998), Allele specific PCR (Hessner *et al.*, 1999), and microarray technology (Erali *et al.*, 2003). These techniques are fast and robust but they require highly expensive instruments and are cumbersome. To make it cost effective and easy for screening large number of samples, an easier technique multiplex PCR-RFLP has also been developed (Koksall *et al.*, 2006), but the present author found it non reproducible. Since Indian populations have

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been found to be infrequent for Prothrombin (G20210A) variant, the present attempt was made to develop a multiplex PCR RFLP detection for diagnosis of FVL and MTHFR C677T mutations using previously published protocols (Frosst *et al.*, 1995 and Bertina *et al.*, 1994). Although these protocols are the most widely accepted and reliable methods for detection of these two variants, but it require separate reaction tubes, twice the ingredients and thrice the working stretch. In contrast to this, our method is fast, robust, single tube reaction with half the operational time and is appropriate to populations devoid of Prothrombin G20210A variant.

MATERIALS AND METHODS

Genomic DNA was isolated from 5ml intravenous blood by salting out precipitation method (Miller *et al.*, 1988). PCR reaction was performed in a total volume of 20 μ L containing 10X buffer A (with 1.5 mM $MgCl_2$), 200 μ M of dNTP's (each), 1.5mM $MgCl_2$, 10pM each of MTHFR primers, 30pM each of FVL primers, 1U Taq DNA polymerase and 50ng of sample DNA, in applied biosystems thermal cycler with the following conditions: An initial denaturation at 94° C for 5 minutes followed by 35 cycles at 94° C for 30 seconds, annealing at 67° C for 30 seconds, 72° C for 30 seconds with a final extension at 72° C for 10 minutes (Table I).

Table I: Primer Sequences

S.No.	Marker	Primer Sequence
1	<i>FVL</i> ^a	
	Forward Primer	5' CAT GAG AGA CAT CGC CTC TG 3'
	Reverse Primer	3' GAC CTA ACA TGT TCT AGC CAG AAG 5'
2	<i>MTHFR C677T</i> ^b	
	Forward Primer	5' TGA AGG AGA AGG TGT CTG CGG 3'
	Reverse Primer	3' AGG ACG GTG CGG TGA GAG TG 5'

(a- Bertina *et al.*, 1994, b-Frosst *et al.*, 1995)

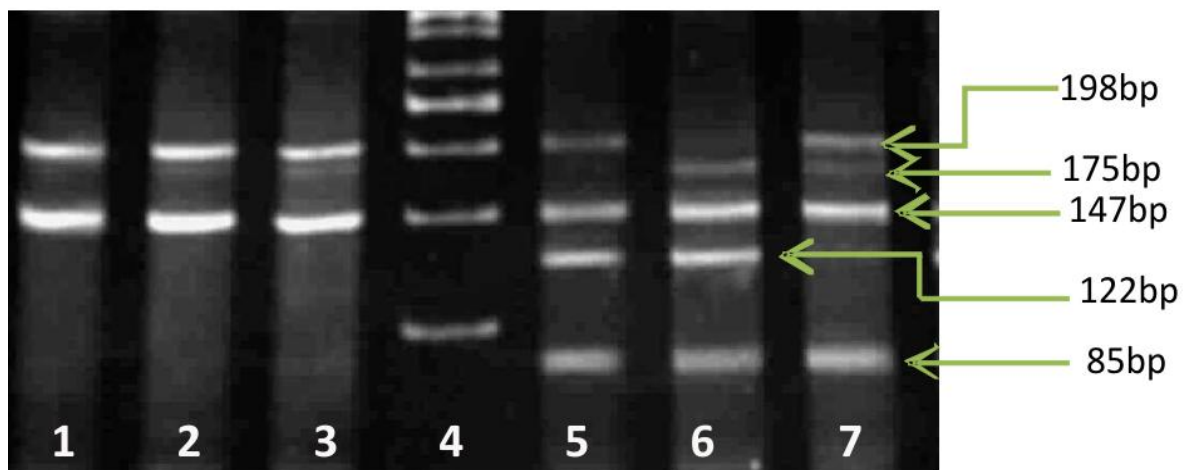


Figure 1: Photograph of 8% polyacrylamide gel showing multiplex PCR RFLP analysis. From left to right, Lane 1-3: Undigested Multiplex PCR amplicon 198bp (MTHFR C677T) & 147bp (FVL); Lane 4: 50bps Ladder; Lane 5: MTHFR normal homozygote & FVL heterozygote; Lane 6: MTHFR mutant homozygote & FVL heterozygote; Lane 7: MTHFR heterozygote & FVL normal homozygote (37bp & 25bp products electrophoresed off the gel). A digested product of 198 bps and 175 bps correspond to the C and T alleles of MTHFR C677T mutation. And a digested product of 122 base pairs and 85 base pairs corresponds to A and G alleles of FVL mutation*.

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***A band size of 147bps corresponds to undigested PCR product of FVL in Hinf I sample digestion.**

Multiplex PCR generated two amplicon sizes of 198bps and 147bps for MTHFR C677T and FVL mutation respectively. 5 µL of PCR product was electrophoresed on 2% agarose gel stained with EtBr to check for non specific amplification. No extra amplifications or non specific bindings were observed during the experimentation which confirms the efficacy and accuracy of the multiplex PCR.

Restriction Digestion

Remaining 15 µL of PCR product was aliquoted equally for digestion with Hinf I and Mnl I enzymes (Fermentas) respectively at 37°C for 1 hour. Both the digested products were mixed and visualized in 8% native polyacrylamide gel under UV light transilluminator (Figure I).

RESULTS AND CONCLUSION

Simultaneous assessment of MTHFR C677T and FVL mutations via multiplex PCR – RFLP method standardized in the present study will be useful as a time and cost effective diagnostic tool for clinicians which will therefore aid in therapeutic treatments.

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