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Research Paper

METHOD VALIDATION FOR DETECTION OF FACTOR V LEIDEN MUTATION BY REAL TIME PCR AND RFLP ANALYSIS

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Factor V Leiden mutation is the most common factor for venous thrombosis and it is associated with the increased risk of pregnancy loss. It's a single point mutation at nucleotide 1691 (G→A) in exon 10 of Factor V gene, which produces an $Arg^{506}Gln$ substitution (R506Q). Although conventional sequencing is a widely used method to detect point mutation but it has several drawbacks. Here we report a rapid and reliable Real Time PCR based method to detect the Factor V Leiden mutation. We have standardized blood DNA isolation with few modifications in SDS based method. The key features are during blood DNA isolation use of hazardous chemicals such as phenol, chloroform: isoamylalcohol etc. was avoided. Without the use of RNase it was possible for us to isolate pure and sufficient amount of DNA which proved to be amenable to Real Time PCR analysis. A desired amplicon of 249 bp was produced. Restriction digestion was carried out using enzyme *Mnl*I which cut at 163 bp and 49 bp.

Keywords: Factor V, RaPC, Point mutation, SYBR green, Real Time PCR, *MnlI*

INTRODUCTION

Coagulation is the complex process by which blood forms clot. Disorders of the coagulation can lead to an increased risk of bleeding. Blood coagulation is under the control of anticoagulant protein present in the plasma or on surface of endothelial cells. Protein C plays a key role in natural anticoagulation (Thanawut *et al.,* 2006). Factor V is a protein of the coagulation system. In contrast to most other factors, it is not enzymatically active but functions as the cofactor converting prothrombin to thrombin. Its deficiency leads to the predisposition for hemorrhage or mostly predispose for thrombosis. Gene for Factor V is located on first chromosome. All human chromosomes come in pairs, during factor V Leiden mutation either one of them can be affected (heterozygous carrier) or both are affected (homozygous carrier). Factor V circulates in plasma as a single chain molecule. It binds to activated platelets and is activated by thrombin (Dahlback *et al.,* 1993).

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Factor V is degraded by activated protein C (aPC), one of the principal physiological inhibitors of the coagulation. Within the blood coagulation cascade the process by which blood clots, Factor V serves as an essential protein cofactor for the efficient generation of thrombin, a primary blood clotting enzyme. Factor V has dual ability to participate as procoagulant cofactor (when active) and anticoagulant (when not activated and binds to aPC) (Dahlback *et al.,* 1999). Factor V is degraded by aPC through sequential proteolysis of three sites (arginine R506, arginine R 306 and arginine R679) in its heavy chain (Bertina *et al.,* 1994 and Dahlback *et al.,* 1994). It was reported for the first time that in the Factor V gene, the nucleotide guanine at position 1691 in exon 10 is replaced by an adenine(G 1691 A). This mutation induced a new codon that changes the R in the position 506 by a glutamine (Q) and consequently a loss in the proteolytic action of aPC (Greengard *et al.,* 1994). With loss of first point of rupture, the reminder breaks much less efficiently, which is called as resistance to activated protein C and this mutation is called as Factor V Leiden. It was found that this mutation and APC resistance is extremely rare in Asian populations (Chan *et al.,* 1996) and it is very common in Caucasian population which is around 1 to 9% (Rees *et al.,* 1995).

The risk of thrombosis increases 5 fold in heterozygous and 30 to 140 fold in homozygous for Factor V Leiden (Bertina *et al.,* 1994 and Dahlback *et al.,* 1994). Coagulation assays for the detection have been described and commercially available. However in some cases they have shown overlap between unaffected and individuals heterozygous for Factor V Leiden. Venous thrombosis annually affects 1 in 10,000 people younger than 40 years and 1 in 1000 persons older than 75 years of age causing significant morbidity and mortality (Nicholaes and Dahlback, 2003). APC resistance is recognized as the most important cause of venous thrombosis present in up to 60% of patients with venous thromboembolism.

Unlike coagulation based assays for the detection for APC resistance, DNA based assays are not affected by pregnancy, therapeutic use of anticoagulants, and use of oral contraceptives or presence of any inhibitors (Koster *et al.,* 1993). With regard to genotyping by Real Time PCR using labeled probes, the greatest advantage is that the signal is very specific (Wittwer CT, 2003). A DNA binding dye such as SYBR green binds to all double stranded products during Real Time PCR, causing increase in fluorescence of the dye. An increase in DNA product therefore leads to an increase in fluorescence intensity and is measured at each cycle, thus allowing qualitative as well as quantitative analysis (Lay *et al.,* 1997). The only disadvantage of SYBR green is that it binds to all double stranded DNA products, sometimes may be some nonspecific products such as primer dimmer. The aim of present study was to standardize a rapid, cost effective and reliable method for the blood DNA isolation to detect the Factor V Leiden mutation with the help of Real Time PCR and RFLP analysis.

MATERIALS AND METHODS

Sample Collection

Patient's blood sample was collected in EDTA tubes from Dr.M.B.Agarwal's Hematology clinic, Mumbai, India.

Primers Used for the Study

Primers (APC 1 and APC 5) were purchased from Sigma Aldrich, USA. Both forward and

reverse primers were reconstituted in Milli- Q water as major stock of 100µM each. Details of primers and their sequences are given in the Table 1.

Enzyme

Enzyme *Mnl*I was purchased from Fermentas, USA and stored at –20^{0}C.

Chemicals and Other Reagents

Extraction Buffers

Buffer A contains 0.32 M Sucrose, 10 mM Tris HCl, 5 mM $MgCl_2$, 0.75% Triton-X-100 and pH was adjusted to 7.6 and Buffer B contains 20 mM Tris HCl, 4 mM EDTA, 100 mM NaCl and pH was adjusted to 7.4

Other reagents: 30% SDS, Proteinase K, 5.3M NaCl, Double distilled water, chilled ethanol and 0.1X TE buffer.

Blood DNA Isolation

To carry out Blood DNA isolation we tried three different protocols.

1. SSC Buffer: (1) Take 1 ml of blood to that add 1 ml of 1X SSC buffer. Centrifuge for 12000 rpm for 2 mins at 4ºC. (2) Discard supernatant and add 1 ml of 1X SSC buffer to the pellet and Mix by inversion. Centrifuge for 12000 rpm for 2 min. at 4ºC. (3) Repeat the above step. (4) Remove the supernatant and to the pellet add 500 µl of 0.2 M Sodium acetate, 25 µl of 10% SDS and 5 µl of Proteinase k (10 mg/ml). Incubate 1 h. at 55ºC. (5) Transfer the supernatant into a new tube and add 500 µl of Phenol/Chloroform/Isoamylalcohol (25:24:1) and mix by inversions. Centrifuge at 4000 rpm for 5 min at 10°C. (6) Transfer the supernatant into a new tube and add 500 µl of Chloroform: isoamyl alcohol (24:1) and mix by inversions. Centrifuge at 4000 rpm for 5 min at 10°C. (7) Repeat the above step 2-3 times to remove the Phenol traces. (8) Transfer the supernatant into a new tube and add 50 µl of 2 M sodium acetate and 500 µl Distilled Ethanol. Invert slowly. DNA will appear in the form of threads. Spin at 5000 rpm for 10 min at 5ºC. (9) Allow the pellet to dry completely, dissolve it into 100 µl of TE buffer till use.

2. SE Buffer: 1. Take 1 ml of blood to that add 3 ml of lysis buffer. Vortex gently and incubate for 30 min in ice. 2. After incubation centrifuge the sample for 12000 rpm for 10 min. at 4ºC. 3. Discard the supernatant and add 1 ml lysis buffer to the pellet. Mix it by inversion. Centrifuge for 12000 rpm for 10 min. at 4ºC. 4. Remove the supernatant and add 0.5 ml of SE buffer to the pellet. Vortex gently and centrifuge at 12000 rpm for 10 min at 4°C. 5. Through the supernatant, add 0.5 ml of SE buffer, 4 µl Proteinase k (10 mg/ml) and 20% SDS. Incubate the sample for 2 h. at 55ºC. Vortex gently at regular intervals of 20 min. 6. After incubation add 0.5ml SE buffer and 1ml phenol and Vortex gently. Centrifuge at 4000 rpm 5 min at 10ºC. 7. Transfer the supernatant into a new tube and add 1 ml of Phenol/ Chloroform/Isoamyl alcohol (25:24:1) and mix by inversions. Centrifuge at 4000 rpm for 5 min at 10°C. 8. Transfer the supernatant into a new tube

Table 1: Primers with Their Sequences Used for Blood DNA Amplification

Oligo Name	Sequence	Length	GC%
Primer APC 1Forward	GACCATACTACAGTGACG	18	50
Primer APC 5Reverse	TGTTATCACACTGGTGCT	18	44

and add 1 ml of Chloroform/Isoamyl alcohol (24:1) and mix by inversions. Centrifuge at 4000 rpm for 5 min at 10°C. 9. Repeat the above step 2-3 times to remove the Phenol traces. 10. Transfer the supernatant into a new tube and add 30 µl of 3 M sodium acetate and 1 ml isopropanol. Invert slowly. DNA will appear in the form of threads. Spin at 5000 rpm for 10 min at 5ºC. 11. Allow the pellet to dry completely, dissolve it into 100 µl of TE buffer till use.

3. Standardized Protocol: 1. Take 1 ml of blood to that add 1 ml of buffer A and 1ml of cold, sterile double distilled water, vortex gently. Incubate on ice for 2-3 mins. 2. Centrifuge at 4000 rpm for 15 mins at 4^0C. 3. Discard the supernatant and to the pellet add 1 ml of buffer A and 3 ml of cold, sterile double distilled water and vortex gently. 4. Centrifuge at 4000 rpm for 10 mins at 4^0C. 5. Repeat the above step till the pellet becomes creamish white in color. 6. Remove the supernatant and to the pellet add 2.5 ml of buffer B and 250 µl of 30% SDS. Vortex vigorously for 30 sec and add 25µl of Proteinase K solution and incubate at 55^0C for 2 h. 7. Mix it well by inversion with an interval of 20 mins. 8. After incubation add 2 ml of 5.3M NaCl solution. Vortex gently for 15 sec. 9. Centrifuge at 5000 rpm for 15 mins at 4^0C. 10. Collect the aqueous layer and add equal volume of ice cold isopropanol. Invert slowly, DNA will appear in the form of threads. 11. Spin the DNA at 5000 rpm for 10 mins at 4^0C. 12. Discard the supernatant and allow the pellet to dry overnight. Next day dissolve the pellet in 100 µl of 0.1X TE buffer.

Agarose Gel Electrophoresis

Isolated DNA was visualized on 0.8% agarose gel containing ethidium bromide (10 mg/ml) in 1X TAE buffer at 60 volts for one and half hour.

Real Time PCR

Real Time PCR was carried out in 7300 Real Time PCR system, Applied Biosystems, USA. All reactions were carried out in 96 well plate Individual reactions contains 12.5µl of 2.5X SYBR Green Master mix, 2.5µl of template DNA, 2.5µl of each forward (APC1) and reverse primer (APC5) and finally volumised to 25µl using sterile Mili-Q water. Negative template control (NTC) was also run along with the DNA samples to check false amplification. Real Time PCR was carried out with initial denaturation for 10 mins at 95^0C followed by 40 cycles of denaturation for 30 sec at 94^0C, annealing for 1 min at 55^0C, extension for 1 min at 72^0C. Finally PCR products were hold for 7 mins at 72^0C. PCR products were visualized on 2% agarose gel containing ethidium bromide (10mg/ml) in 1X TAE buffer at 100 volts for half an hour.

Restriction Fragment Length Polymorphism

Restriction digestion of PCR product was carried out with a final volume of 15 µl containing 5 µl of PCR product, 8 µl of deionised water, 1µl of 10X buffer G and 1 µl of enzyme *Mnl*I. Samples were incubated at 37^0C for 2 h. After two hours enzyme activity was inactivated by incubating at 65^0C for 20 min. RFLP pattern was visualized on 2% agarose containing ethidium bromide (10 mg/ml) in 1X TAE buffer at 80 volts for 40 min.

RESULTS AND DISCUSSION

The isolation of genomic DNA from blood typically involves digestion of nuclei with a combination of Proteinase K and Sodium Dodecyl Sulphate (SDS) followed by deproteinization with organic reagents such as Phenol and Chloroform (Grimberg *et al.,* 1999). To isolate pure DNA from Blood samples we tried three different protocols. During extraction, we encountered many hurdles

such as interference of hemoglobin, proteins and other components of blood. Inconsistent results were observed when we used SSC buffer for DNA isolation. Either very less DNA was isolated or there was no DNA yield. In case of SE buffer, though DNA was isolated but the pellet was reddish in color and gelatinous which was very difficult to get dissolve in TE buffer. Electrophoresis pattern showed presence of RNA and proteins along with DNA (Table 2). On the contrary, our standardized protocol yielded pure and good amount of DNA (Figure 1, depicted with arrow). There was no protein or RNA contamination associated with the isolated DNA.

To isolate pure blood DNA we made few modifications in the protocol,

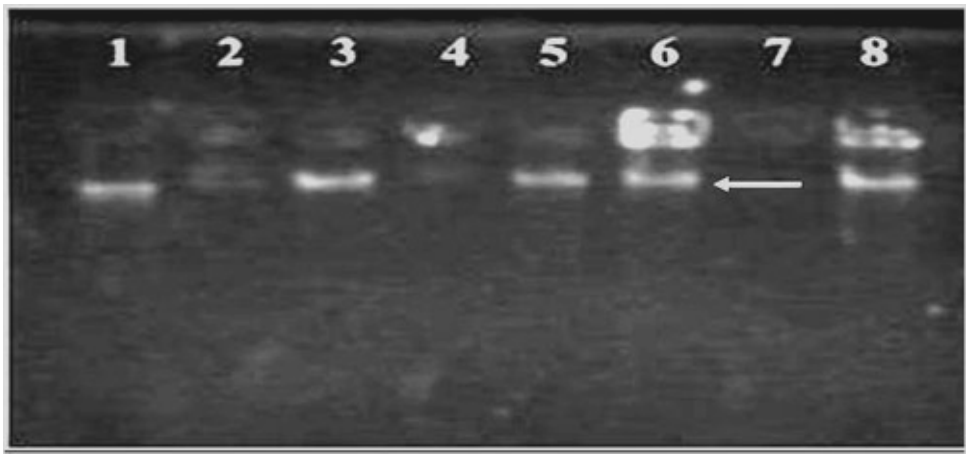
1. During first step we found use of Milli-Q water instead of distilled water which removed hemoglobin more efficiently. Hence subsequent washing were reduced.
2. To remove proteins, we used 15, 25, 50 µl of Proteinase K for 1 and 2 hours incubation and observed that incubation with 25µl for two hours removed proteins completely.
3. It was observed that chilled isopropanol precipitated more and pure DNA than that of chilled ethanol.

The key feature of the protocol was we neither used phenol nor chloroform: isoamylalcohol (24:1) for subsequent washings which avoided the traces of phenol throughout the procedure. During initial step we used sucrose which created osmotic pressure in the red blood cells causing rapid removal of hemoglobin and decolonization of the pellet. It also helped to lyse erythrocytes (Heller *et al.,* 1991). With these modifications we

Table 2: Different Protocols Used for Blood DNA Isolation

Protocol	Result	Remarks
1. SSC buffer	No DNA isolated	No band was observed
2. SE buffer	Very less DNA was isolated	A feeble band with lot of RNA and protein contamination
3. Standardized protocol	DNA was isolated consistently	A sharp band was observed. DNA was suitable for Real Time PCR analysis

Figure 1: Genomic DNA of Blood Resolved on 0.8% Agarose Gel

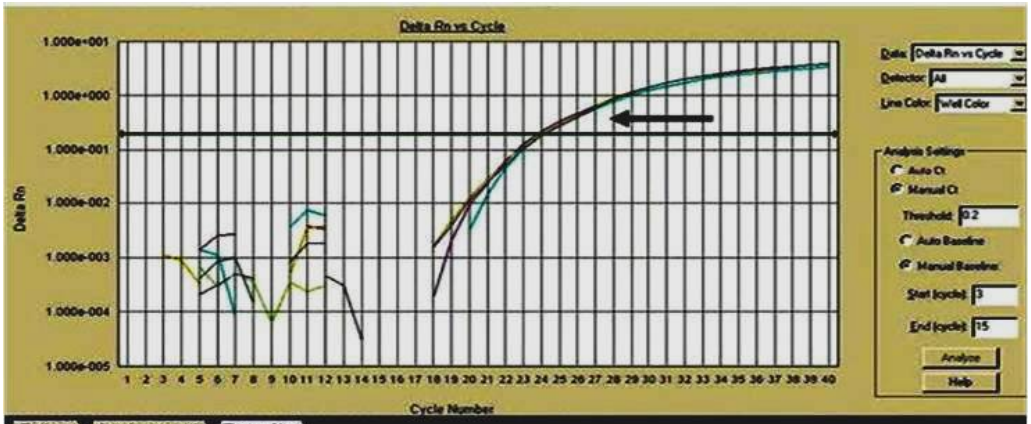


obtained pure and large amount of blood DNA (Figure 1, lane 5 and 6).

Real Time PCR Analysis

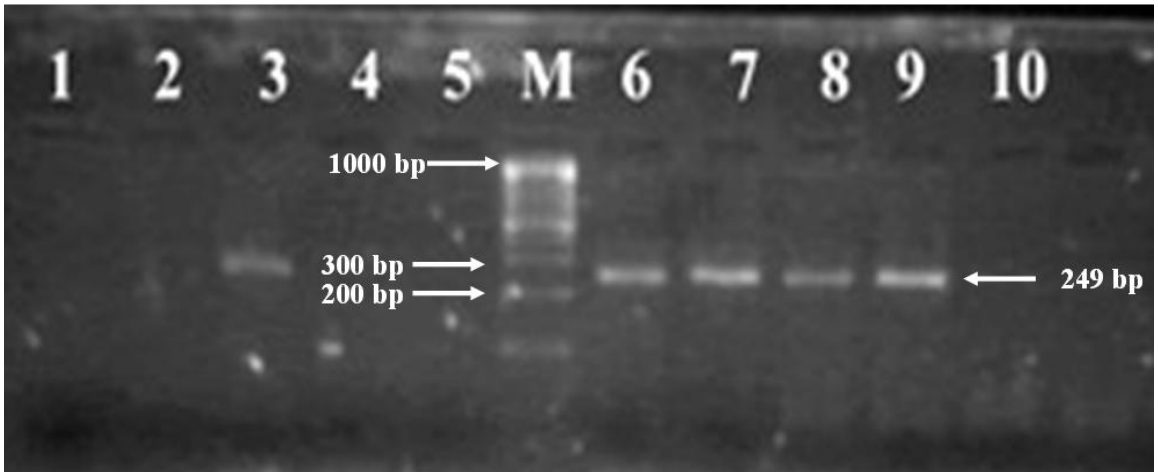
By Real-time PCR the desire segment of DNA, a flaking region of 1691 for Factor V gene mutation was amplified. Proper amplification curve was observed by Real Time PCR analysis (Figure 2, depicted with black arrow). From the Ct values we can say that patient's DNA was amplified properly and in case of NTC, the Ct value was undetermined confirming that there was no false amplification during reaction (Figure 2). A desired product of 249 bp was visualized on 2% agarose gel (Figure 3, lane 6, 7, 8 & 9).

Figure 2: Amplification Curve of Blood Samples Using Real Time PCR



Well	Sample Name	Detector	Task	Ct	StdDev Ct	Quantity	Mean Qty	StdDev
G1	Normal DNA 1:	APC	Unknown	24.1411				
G2	Normal DNA 1:	APC	Unknown	23.7628				
G3	P DNA 1:2	APC	Unknown	24.0016				
G4	P DNA 1:5	APC	Unknown	24.1896				
G5	NTC	APC	NTC	Undet.				
G6	NTC	APC	NTC	Undet.				

Figure 3: Real Time PCR Product of 249 bp Resolved on 2% Agarose Gel



Restriction Endonuclease Digestion Analysis

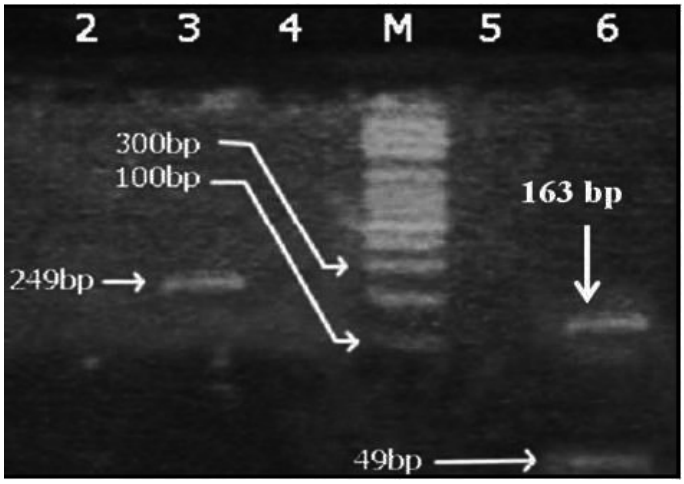
As mentioned earlier, restriction digestion of PCR product was carried out using enzyme *Mnl*I. In Figure 4, lane 6 shows restriction digestion products of molecular weight 163 bp between 100 to 200 bp and 49 bp towards the end (depicted with arrows).

DISCUSSION

Functional resistance of coagulation factor V against the anticoagulant activity of aPC was first described by Dahlback *et al*. in 1993. This protein is incapable of proteolyzing and inactivating factor V to prevent thrombosis. Factor V Leiden which is a genetic mutant of coagulation factor V confers a thrombophilic state, there are other genetic risk factors do develop venous thrombosis (Chegeni *et al.,* 2005). Protein S, protein C and antithrombin deficiency are the examples of important genetic risk factors. Acquired risk factors such as obesity, cancer, contraceptive pills, pregnancy and smoking also play an important role in development of venous thrombosis (Chegeni *et al.,* 2005). Though conventional sequencing is widely used method for the mutational analysis such as point mutation SNP's (Single nucleotide polymorphism) and single strand conformation polymorphism (Margaglione *et al.,* 1996 and Corral *et al.,* 1997) or heteroduplex technology (Bowen *et al.,* 1997), but initial reports on factor V leiden mutation used PCR amplification of the region of factor V gene containing base substitution followed by restriction digestion with *Nla III* (Beauchamp *et al.,* 1994) and *Hind III* (Gandrille *et al.,*1995) were more promising.

However this method is laborious, technically demanding and expensive. To overcome these difficulties it was decided to standardize a rapid, reliable Real Time PCR based method to detect factor V Leiden mutation. Here we have detected factor V Leiden mutation using SYBR green dye I melt curve analysis (Wittwer CT, 2003). To begin with, first we have standardized rapid blood DNA isolation without the use of hazardous chemicals such as Phenol and Chloroform: isoamylalcohol etc. Even no RNase was required as isolated DNA was very much purified (Figure 1, lane 5 and 6). Bands were sharp and consistent. For Real Time PCR analysis we have initially followed the

Figure 4: Restriction Fragment Analysis of PCR Product Resolved on 2% Agarose Gel



method described Chegeni *et al,* 2005. But we couldn't get satisfied amplification curve. Hence we modified the PCR cycle as initial denaturation was carried out for 10 min instead of 5 min at 95^0C. At the same time we carried out 40 cycles instead of 30 and final extension for 7 min at 72^0C. This gave us the proper amplification curve for normal as well as patient's blood DNA.

As discussed earlier, during SYBR green dye I analysis there are chances of formation of non specific products such as primer-dimer. Hence in our study we have run NTC (Non template control) parallel with that of sample DNA. As we can see from Figure 2, graph table well no G5 and G6, Ct values was undetermined confirming that there was no false amplification and for normal and patient's sample Ct values were in the range of 23 to 24 as a proper amplification curve was observed (Figure 3 depicted with black arrow). The advantage of this method is that fluorescence intensity gives amplification curve that distinguishes between sample DNA and NTC. Gandrille et al 1995 detected factor V Leiden mutation using restriction endonuclease Hind *III* producing fragments of 33 and 92 bp. Central to the approach presented is the use of single restriction endonuclease (*Mnl*I) recognition site in the desired amplicon to assess the completeness of restriction endonuclease digestion which can distinguishes heterozygous from homozygous. As reported earlier, digestion with Mn*l*I results in fragments of 200, 163,49 and 37 bp when the mutation is not present and fragments of 37,49 and 163 bp when it is present in the heterozygous configuration and 49 and 200 bp with that of homozygous condition (Schalasta and Schmid, 1997). In our study digestion with Mn*l*I produced the amplicon of 163 and 49 bp confirming that it was a sample of heterozygous patient (Figure 4, Lane 6).

CONCLUSION

To conclude, we can say the method we developed is very fast, reliable and less time consuming for detection of factor V Leiden mutation using Real Time PCR and RFLP analysis. We couldn't get much blood samples of patients suffering from factor V Leiden mutation, but still this method can be applied for large number of samples suffering from various genetic disorders and at the same time to variety of targets.

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