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Molecular detection of inherited haematological disorder of human

Sanjay Kumar Pandey
Centre for Biotechnology studies, Awadhesh Pratap Singh University, Rewa-486003, M.P., (INDIA)
E-mail: pandeysanjaybt@rediffmail.com

ABSTRACT

A human disease studies on molecular level and its diagnostics are result of our approach towards our aim to look after patients in their absolute genetic needs from counselling to DNA profiling for disease prevention, by taking entire genetic tests from hereditary diseases to infectious diseases from introduction of individualized medicines to prenatal diagnosis from noninvasive methods. Molecular Diagnosis of disease is very useful way to detect abnormalities in DNA level. The technique is rapid, cost effective, accurate and gold standard. Various diseases i.e. Infectious (Viral, Bacterial, Fungal), Inherited and malignancies can be detected in early stage and medication can be started that save time, money and life.

KEYWORDS

PCR;
Genotyping;
DNA;
Molecular Diagnosis.

INTRODUCTION

Genetic diseases are inherited through generations from parents to their progeny, though there is treatment available to these genetic disorders but they are in their initial phase and are not time tested thus not reached to gold standards so as of now only its proper management is done to control and prevent it. For its prevention genetic counselling, Pre marital counselling and prenatal diagnosis process are only the measures available. But these investigations are not available to common mass people as its too costly and due to huge population burden. Genetic diseases include thalassemia, sickle cell anemia, hemophilia, thrombophili etc. which take very large investment in its management, which include frequent blood transfusion to the patient which is not possible for poor rural background people which leads to patient's mortality. In heterozygous condition i.e trait cases by genetic investigations we can avoid the marriage between traits by medical counselling by which homozygous conditions of the diseases are avoided and it remain traits in that conditions the person is healthy as this condition is asymptomatic. Following human diseases can be identified using molecular analysis methodology.

(A) Hematological Disorders

Hemoglobinopathies and thalassemia (HbS, HbE,HbD and β-thalassemia,δ α-thalassemia, δ β-thalassemia), Hemophilia A and B (Factor VIII and IX deficiency), Thrombosis, G6PD, HFE, XMN1,MTHFR, FV Leiden

(B) Rare disorders

Duchenne and Becker muscular dystrophy, Cystic fibrosis, A1-antitrypsin deficiency, Familial hypercholesterolemia, apolipoprotein C-Il deficiency,

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Huntington'schorea, Tay-Sachs disease.

(C) X-linked diseases based upon Y chromosome/ Other-Diabetes

Huntington *disease*, Lyme *Disease*, Kawasaki *disease*, Chagas' *disease*, pertussis, multiple sclerosis, Human Alzheimer's *Disease*, Whipple's *disease*, Aleutian *disease*.

(D) Viral disease

Hepatitis A, B,C, HIV, H5N1 bird flu, herpes simplex, papillomavirus, BK Virus,Cytomegalovirus, Enterovirus, Epstein-Barr Virus, Herpes Simplex, Human Metapneumovirus, Human Papillomavirus, Influenza Virus A & B, Influenza Virus H1N1, Norovirus, Parvovirus B 19, Varicella-Zoster, West Nile Virus.

(E) Bacterial disease

Mycobacterium tuberculosis, *Chlamydia pneumoniae* in C*SF,* C. *pneumoniae* in CSF, Pulmonary Tuberculosis**,** Helicobacter pylori (Cryptosporidium spp.) Cryptosporidium, staphylococcus,*Mycoplasma pneumoniae, Neisseria gonorrhoeae, Streptococcu. Mycobacterium, tuberculosis, Legionella* Species, *Gardnerella vaginalis Clostridium difficile, Chlamydia trachomatis, Bartonella, Borrelia specie.*

(F) Fungal Disease

Coccidioides immitis, Candida species, Blastomyces dermatitidis Histoplasma capsulatum,Toxoplasma gondii, Trichomonas vaginalis

(G) Malignancies

Leukemia (ALL, AML.CML.CLL), lymphoma, colon cancer other cancer,

(H) Early detection of cardiac risk Marker

Stroke, *Atherosclerosis*, DVT.

(I) Forensic analysis

Paternity Test, Genetic Predisposition Test, DNA Profiling Test, Ancestral Origins Test Immigration Test

Here I am presenting simplest methodology of molecular detection of inherited haematological disorder of human by using PCR in haematological disorder forever.

MATERIAL AND METHOD

Sample -Venous blood (1 ml)

DNA Extraction - Phenol chlorophorm and /or Kit Method

Genotypic Detection

1. Genotypic detection of sickle cell by allele specific PCR[1]

Primer sequence

WT-AS (52 -ATG GTG CAC CTG ACT CCT GA-32) WT- Fw control
CP517 (52 -CCC CTT CCT ATG ACA TGA ACT-32) Rw control
MUT-AS (52 -CAG TAA CGG CAG ACT TCT CCA-32) Fw mutant
MUT-CP267 (52 -GGG TTT GAA GTC CAA CTC CTA-32) Rw mutant

PCR program for amplification

- Incubate at 95^0C for 00 :02:00
- Incubate at 95^0C for 00 :00:30
- Incubate at 65^0C for 00 :00:30
- Incubate at 72^0C for 00 :00:30
- Cycle to step 2 for 30 more times.
- Incubate at 72^0C for 00 :05:00
- Incubate at 10^0C for 00 :15:00

Reaction mixture

S.No.	Chemical	Amount(28µl)
1.	1x Frankfurt buffer	20µl
2.	Fw primer	0.5µl
3.	Rw primer	0.5 µl
4.	DMSO	3 µl
5.	Taq polymerase	0.5 µl
6.	DNA sample	2.5 µl

Confirmation of amplification - Amplified products were run on 2% agarose gel.

2. Detection of alpha thalassemia by Gap- PCR

(I) - $\alpha^{3.7\,Kb}$ deletion[2]

Primer Sequence

A- 5'-CTTTCCCTACCCAGAGCCAGGTT-3'
B- 5'-CCCATGCTGGCACGTTTCTGAGG-3'
C- 5'-CCATTGTTGGCACATTCCGGGACA-3'

PCR program for amplification

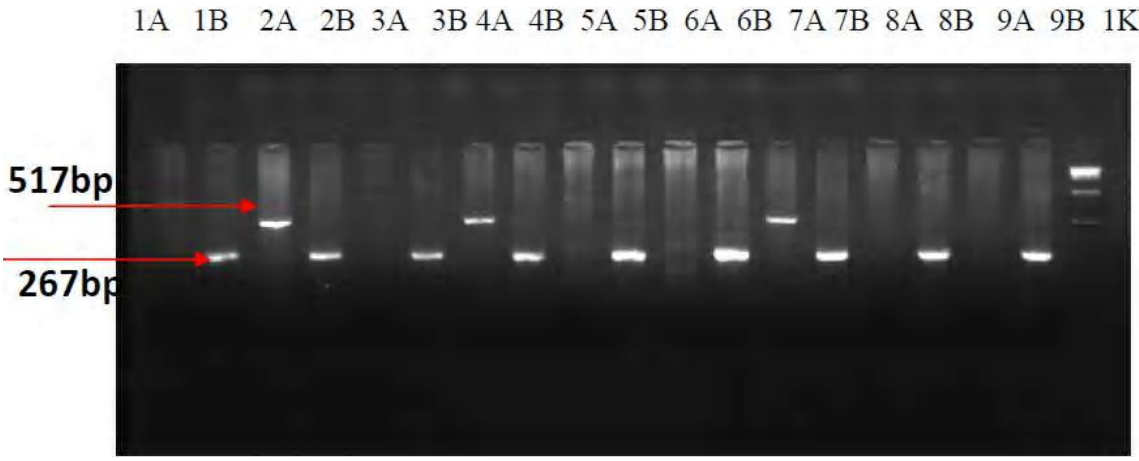

Figure 1 : Gel picture of sickle cell gene (Lane 1,3,5,6,8,9 homozygous and 2,4,7 heterozygous)

- Incubate at 95^{0}C for 00 :02:00
- Incubate at 95^{0}C for 00 :00:30
- Incubate at 60^{0}C for 00 :00:30
- Incubate at 72^{0}C for 00 :00:30
- Cycle to step 2 for 30 more times.
- Incubate at 72^{0}C for 00 :05:00
- Incubate at 10^{0}C for 00 :15:00

Reaction mixture

S.No.	Chemical	Amount(27µl)
1.	1x Frankfurt buffer	22µl
2.	Fw primer	0.5µl
3.	Rw primer	0.5µl
4.	MgCl2	1 µl
5.	Taq polymerase	0.5 µl
6.	DNA Sample	2.5 µl

Confirmation of amplification: Amplified products were run on 2% agarose gel.

(ii) α $^{4.2}$kb deletion[2]

Primer sequence

D- 5'-CCTTCCTCTCACTTGGCCCTGAG-3'
E- 5'-CCCTGGGTGTCCAGGAGCAAGCC-3'
F- 5'-GGCACATTCCGGGACAGAGAGAA-3'

PCR –program for amplification

- Incubate at 95^{0}C for 00 :06:00
- Incubate at 94^{0}C for 00 :01:00
- Incubate at 58^0 C for 00 :01:00
- Incubate at 72^{0}C for 00 :01:00
- Cycle to step 2 for 35 more times.
- Incubate at 72^{0}C for 00 :10:00
- Incubate at 10^{0}C for 00 :15:00

Confirmation of amplification: Amplified products were run on 2% agarose gel.

(iii) —^{SEA} délétion[3]

Primer séquence

A. 5'-GCGATCTGGGCTCTGTGTTCT-3'
B. 5'-GTTCCCTGCCCCGACACG-3'

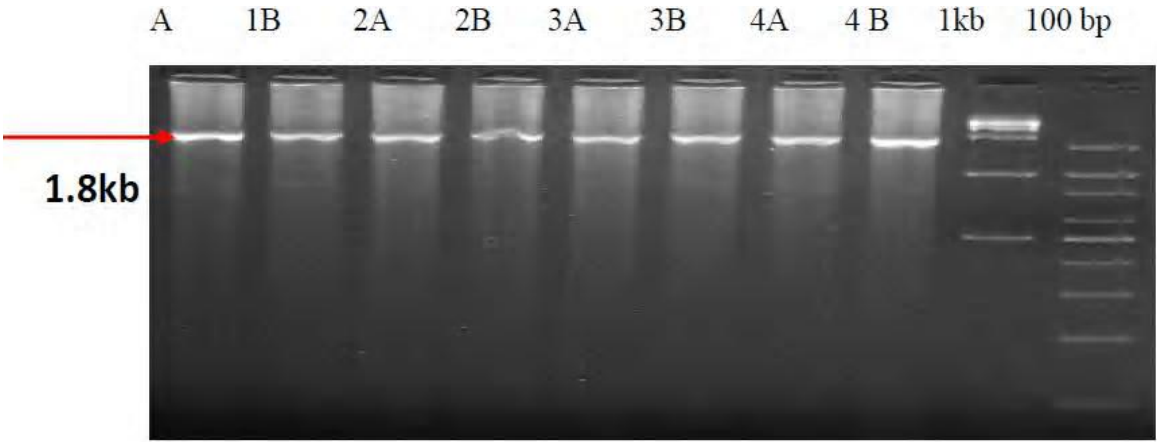

Figure 2 : Lane 1-4 Heterozygous (capital letters is a mutant set while small letters is normal set)

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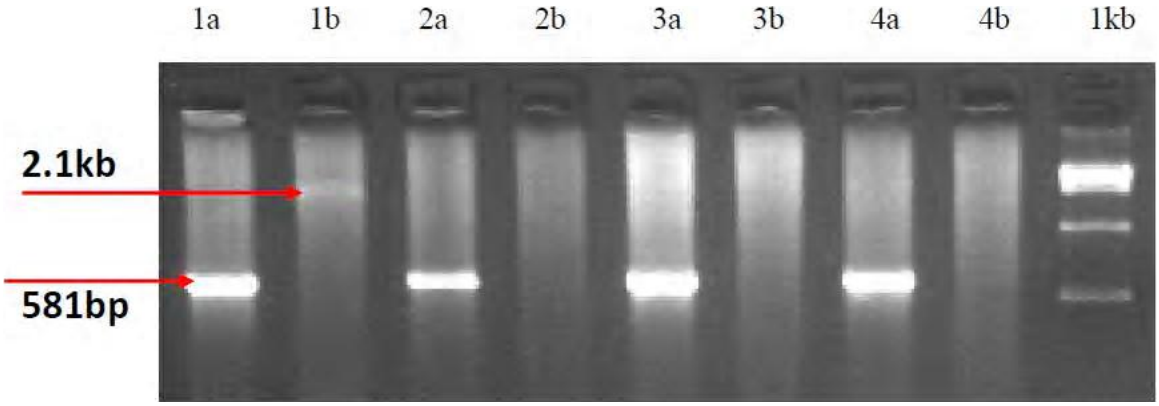

Figure 3 : α 4.2 kb deletion (Lane 1a 1b heterozygous)

Reaction mixture

S.No.	Chemical	Amount(28µl)
1.	1x Frankfurt buffer	20µl
2.	Fw primer	0.5µl
3.	Rw primer	0.5 µl
4.	DMSO	3 µl
5.	Taq polymerase	0.5 µl
6.	DNA sample	2.5 µl

C. 5'-ACTGCAGCCTTGAACTCCTG-3'

PCR –program for amplification

- Incubate at 95^{0}C for 00 :05:00
- Incubate at 94 ^{0}C for 00 :01:00
- Incubate at 68^{0}C for 00 :01:00
- Incubate at 72^{0}C for 00 :02:30
- Cycle to step 2 for 24 more times.
- Incubate at 72^{0}C for 00 :05:00
- Incubate at 4^{0}C for 00 :10:00

Confirmation of amplification: Amplified products were run on 2% agarose gel.

(iv)—^{SA} deletion[4,5]

Primer sequence

α^{52} 5'-CTCTTTCTCTCGGAGCCCTT-3'Fw
α^{53} 5'-ACTCGAGCTACCCCAAGGAT-3'Rw
$\alpha^{2/3.7\text{-}F}$ 5'-CCCCTCGCCAAGTCCACCC-3'Fw
$\alpha^{7/20.5\text{-}R}$ 5'-AAAGCACTCTAGGGTCCAGCG-3'Rw

Confirmation of amplification: Amplified products

Reaction mixture

S.No.	Chemical	Amount(26µl)
1.	1x Frankfurt buffer	20µl
2.	Fw primer	0.5µl
3.	Rw primer	0.5 µl
4.	DMSO	2 µl
5.	Taq polymerase	0.5 µl
6.	DNA sample	2.5 µl

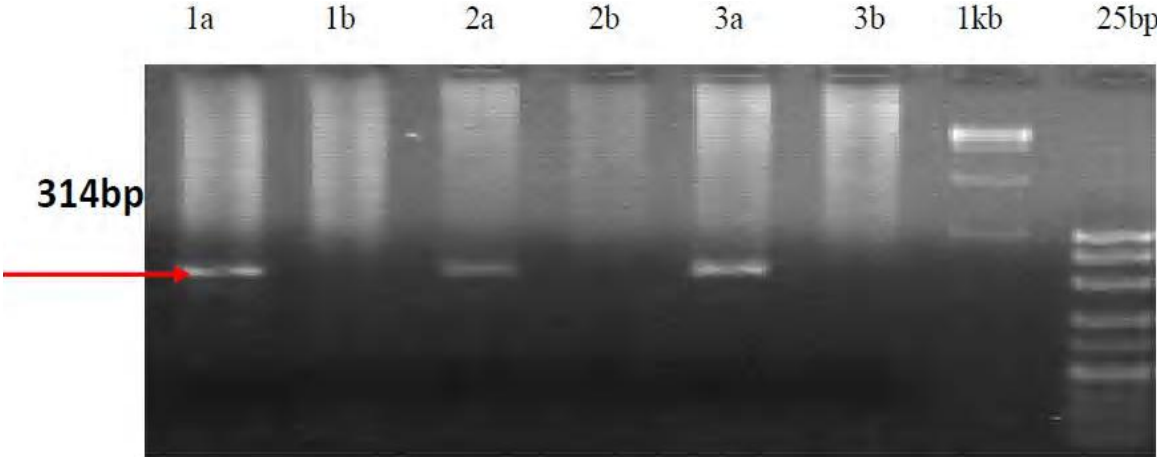

Figure 4 : South East Asian deletion (All normal)

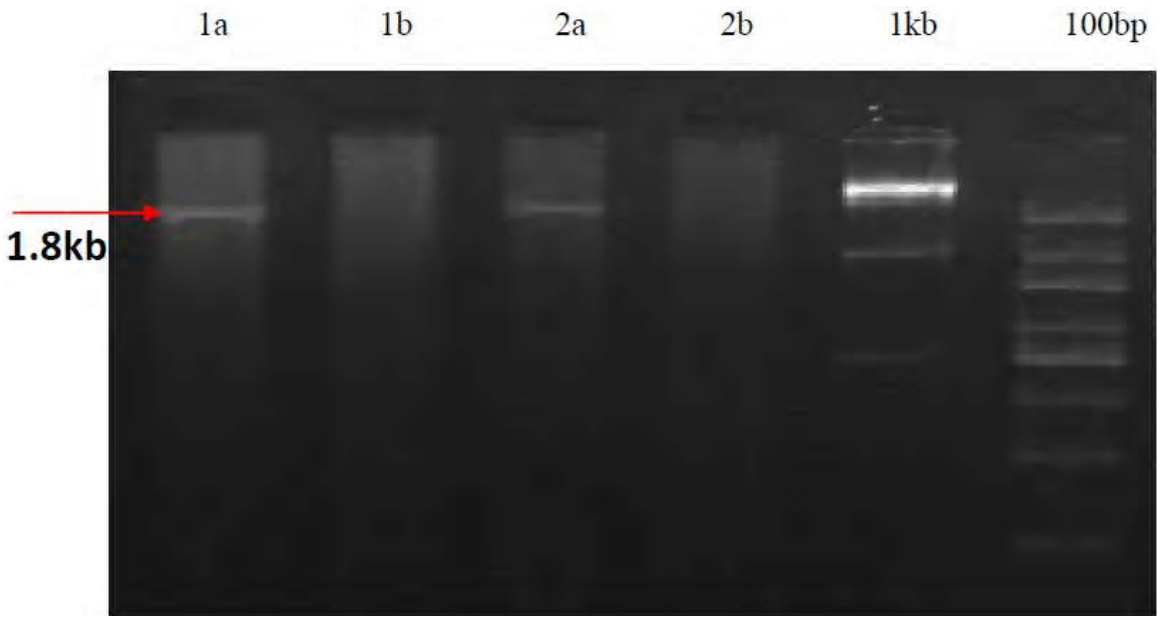

Figure 5 : South African deletion (All normal)

Reaction mixture (Common for all β-thalassemia mutation)

S.No.	Chemical	Amount (26µl)
1.	1x Frankfurt buffer	22µl
2.	Fw primer	0.5µl (5pmol)
3.	Rw primer	0.5µl
4.	Taq polymerase	0.5 µl
5.	DNA sample	2.5 µl

were run on 2% agarose gel.

(v)Detection of α triplication (anti-3.7 del)[6]

Primer Sequence

Fw 5'-CCCTCCCCGAGCCAAGCCTCCTCCC-3'
Rw 5'-GGGAGGCCCATCGGGCAGGAGGAAC-3'

PCR program for amplification

- Incubate at 95^{0}C for 00 :05:00
- Incubate at 94^{0}C for 00 :01:00
- Incubate at 65^{0}C for 00 :01:00
- Incubate at 72^{0}C for 00 :01:00
- Cycle to step 2 for 30 more times.
- Incubate at 72^{0}C for 00 :05:00
- Incubate at 10^{0}C for 00 :15:00

Confirmation of amplification: Amplified products were run on 2% agarose gel.

3. Detection of β-thalassemia mutation –ARMS - PCR

Beta thalassemia mutation screening[7]

PCR –program for amplification (Common for all β-thalassemia mutation)

- Incubate at 93^0 C for 00 :05:00

Reaction mixture

S.No.	Chemical	Amount (28µl)
1.	1x Frankfurt buffer	20µl
2.	Fw primer	0.5µl
3.	Rw primer	0.5µl
4.	MgCl2	1 µl
5.	DMSO	3 µl
6.	Taq polymerase	0.5 µl
7.	DNA sample	2.5 µl

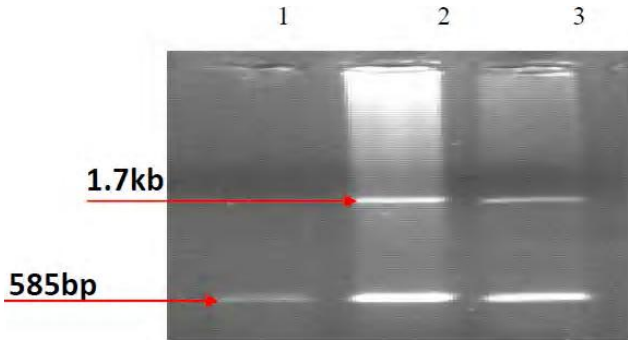

Figure 6 : Alpha triplication (Lane 2,3 positive for anti 3.7 kb del.)

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Primer sequence

Mutation	Primer sequence
IVS1-5(G-C)	5’ CTCCTTAAACCTGTCTTGTAACCTTGTTAG-3’Fw
IVS1-1(G-T)	5’ TTAAACCTGTCTTGTAACCTTGATACGAAA-3’ Fw
Cd8/9(+G)	5’CCTTGCCCCACACGGCAGTAACGGCACACC-3’Fw
Cd 41/42(-CTTT)	5’GAGTGGACAGATCCCCAAAGGACTCAACCT-3’Fw
	5’ ACCTCACCCTGTGGAGCCAC-3’ Common reverse
-88(C-T)	5’TCACTTAGACCTCACCCTGTGGAGCCTCAT3’ Fw
	5’CCCCTTCCTATGACATGAACTTAA3’Rw
Cap Site+1(A-C)	5ATAAGTCAGGGCAGAGCCATCTATTGGTTC3’Fw
	5’CCCCTTCCTATGACATGAACTTAA3’Rw
Internal control for β Chain	5’GAGTCAAGGCTGAGAGATGCAGGA-3’fw
	5’CAATGTATCATGCCTCTTTGCACC-3’rw
619 bp del*	* Automated 242 bp product seen instead of 861 bp product if 619 bp deletion presence during above mutation screening

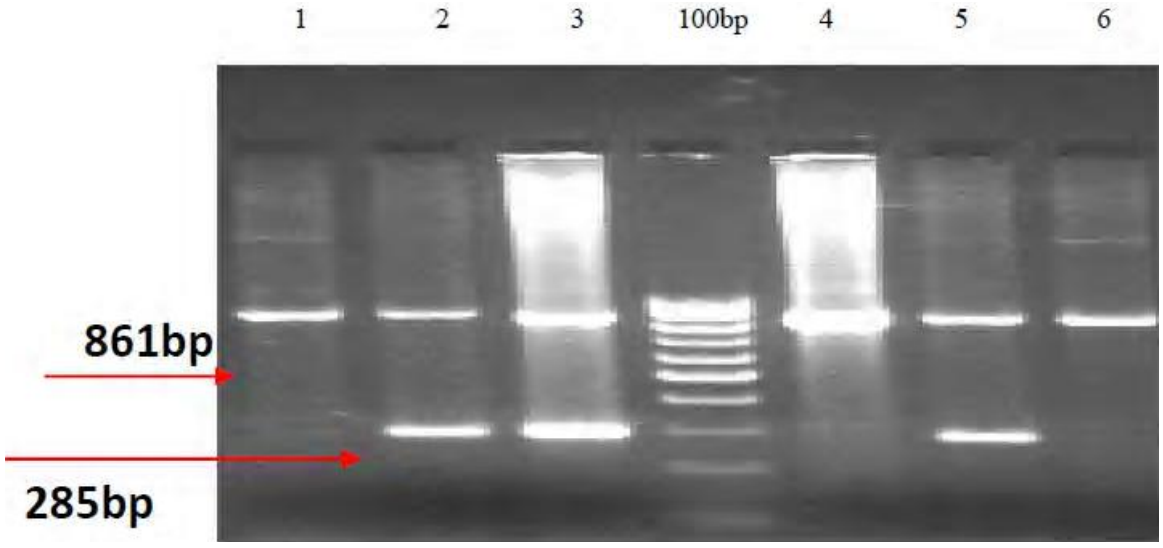

Figure 7 : Beta thalassemia mutation (Lane 2, 3, 5 positive for IVS 1-5)

- Incubate at 93^{0}C for 00 :01:00
- Incubate at 66^{0}C for 00 :02:00
- Cycle to step 2 for 23 more times.
- Incubate at 93^{0}C for 00 :01:00
- Incubate at 66^{0}C for 00 :03:00
- Incubate at 72^{0}C for 00 :10:00
- Incubate at 10^0 C for 01 :00:00

4. Detection of Xmn1 polymorphism[8]

Primer sequence

Fw 5'-AACTGTTGCTTTATAGGATTTT-3'
Rw 5'-AGGAGCTTATTGATAACTCAGAC-3'

PCR program for amplification

- Incubate at 95^{0}C for 00 :05:00
- Incubate at 94 ^{0}C for 00 :01:00
- Incubate at 72^{0}C for 00 :01:00
- Cycle to step 2 for 30 more times.

Reaction mixture (Common for all β-thalassemia mutation)

S.No.	Chemical	Amount (26µl)
1.	1x Frankfurt buffer	22µl
2.	Fw primer	0.5µl (5pmol)
3.	Rw primer	0.5µl
4.	Taq polymerase	0.5 µl
5.	DNA sample	2.5 µl

Reaction Mixture

S.No.	Chemical	Amount(27µl)
1.	1x Frankfurt buffer	20µl
2.	Fw primer	1µl
3.	Rw primer	1 µl
4.	Taq polymerase	0.5 µl
5	DNA sample	2.5ul

- Incubate at 60 ^{0}C for 00 :01:00
- Incubate at 72^{0}C for 00 :02:00
- Cycle to step 2 for 35 more times.
- Incubate at 72^{0}C for 00 :05:00
- Incubate at 4^{0}C for 00 :10:00

Restriction Digestion

- Incubate at 72^{0}C for 00 :05:00
- Incubate at 4^{0}C for 00 :10:00

Confirmation of amplification: Amplified products were run on 2% agarose gel.

PCR –program for amplification (Common for 5 SNPs)

- Incubate at 94 ^{0}C for 00 :01:00

Restriction Digestion Xmn1

S.No.	Chemical	Amount(50µl)
1.	Sterile water	18µl
2.	PCR Product	10µl
3.	Buffer	2µl
4.	Enzyme-Xmn-1	1 µl

Product Incubate at 37°C for overnight

⇩

Confirm on 3% agarose gel

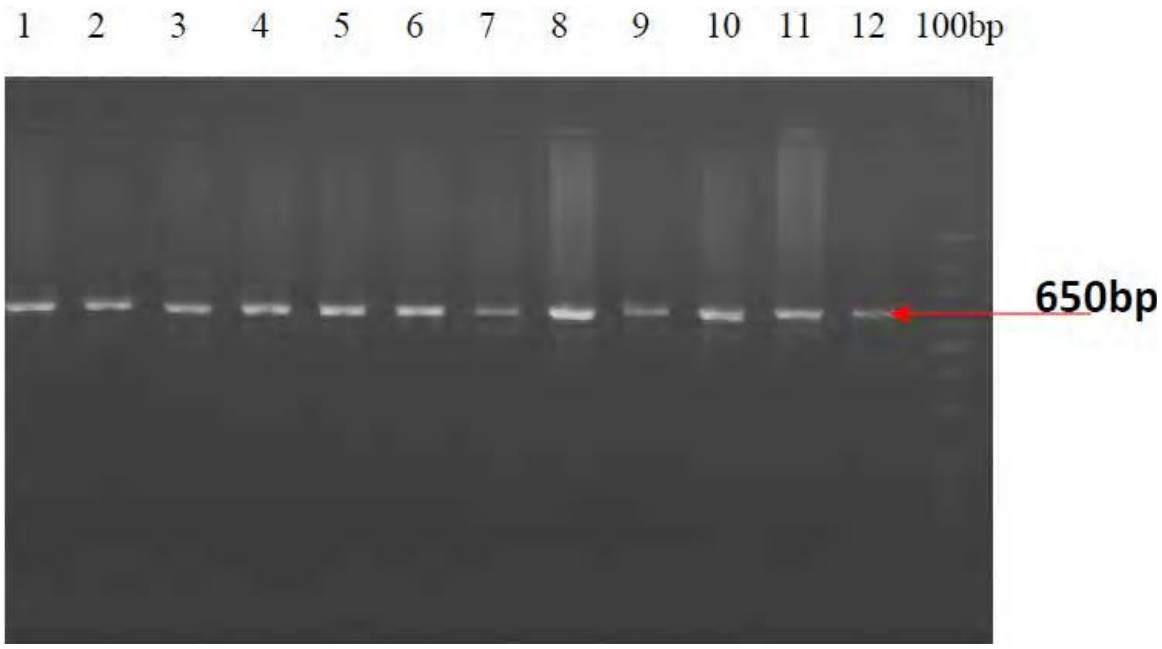

Figure 8 : Check gel for *XMN1*

PCR – program for amplification (Common for 5 SNPs)

- Incubate at 94 ^{0}C for 00 :01:00
- Incubate at 60 ^{0}C for 00 :01:00
- Incubate at 72^{0}C for 00 :02:00
- Cycle to step 2 for 35 more times.

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5. Primer sequence and restriction digestion of BMP6 gene SNPs(Sickle osteeonecrosis) (Pandey et.al.2012)[9]

Gene	SNPs	Primer sequence	Restriction enzyme
BMP6	rs73719353	5'-GCTCCTTTGCACTTCGCTGT-3' F 5'-AGGCTCTGCTGAGCTCCTAC-3' R	Sfc1
	rs73719341	5'-TGAACTTCCCATTCCCCTCT-3' F 5' ATAAAATTAGCATTGATCCA 3' R	BccI
	rs73719318	5'- CAGGT GCTGTGCAAC TTCTT -3'F 5'-AGAGGGCACCATGGTTGCCT-3'R	HpyCH4V
	rs73381662	5'- CTGAGATTCAATTAGGCCCA -3'F 5'-TAAAGAACAGCAAAAGTCTG-3'R	ScrFI
	rs73381650	5'-CACATAAAGATTGCTGCATT-3'F 5'- TAGTAATCCTAAAAATGGGA-3'R	AflIII

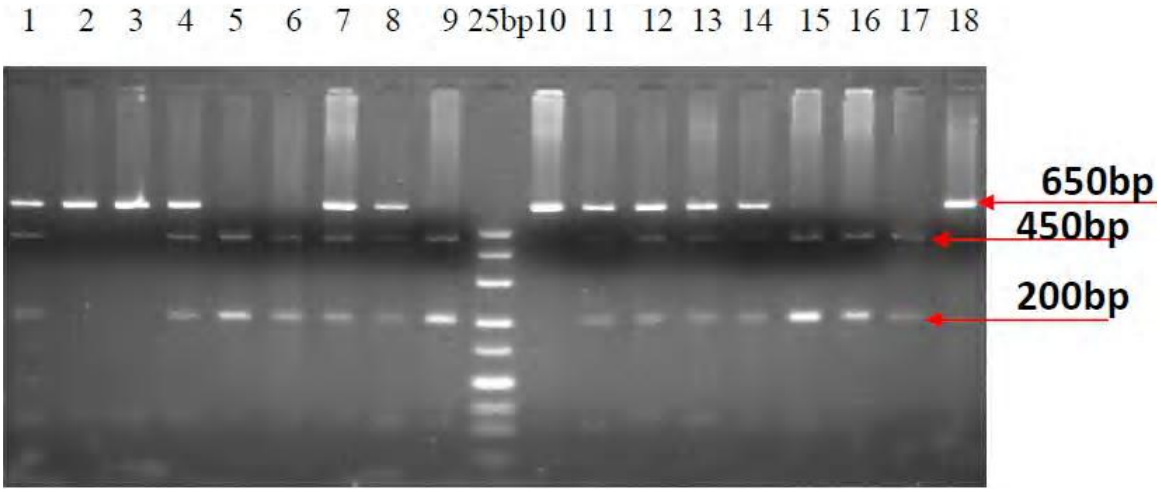

Figure 9 : Restriction digestion with Xmn-1 enzyme

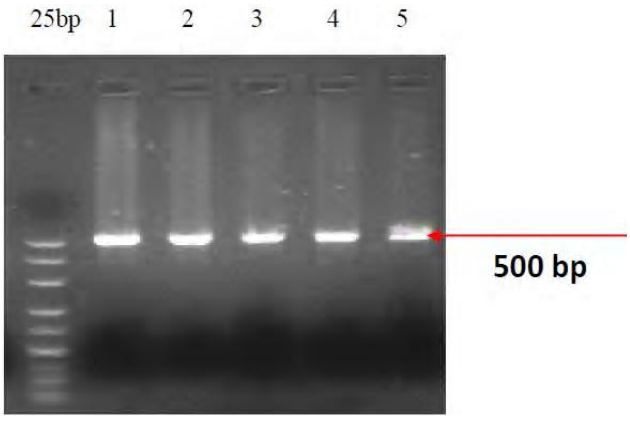

Figure 10 : Check gel of rs73719353

- Incubate at 72^{0}C for 00 :05:00
- Incubate at 4^{0}C for 00 :10:00

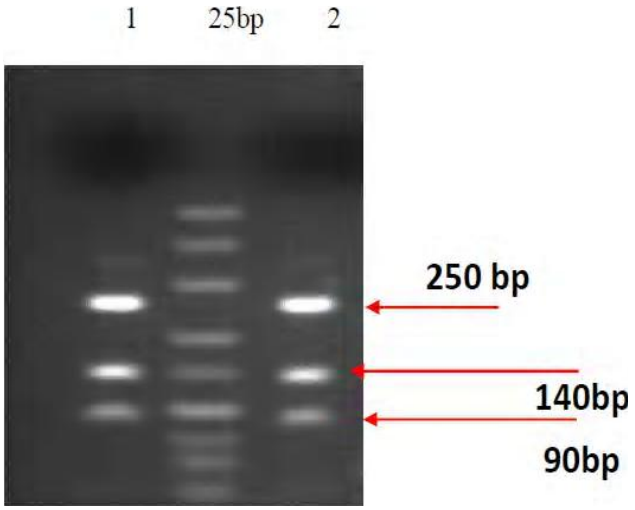

Figure 11 : Restriction digestion of rs73719353 with Sfc1

SNPs genotype of BMP6 and restriction product size

SNPs	Genotype	Product size: Total product size	Product size: Restriction fragments product size
	GG-/-		340,140,20bp
rs73719353	GT-/+	500bp	340,250,140,90,20bp
	TT+/+		250,140,90,20bp
	GG-/-		212,188bp
rs73719341	GA-/+	400bp	212,188,150,39bp
	AA+/+		212,150,38bp
	CC-/-		207,89bp
rs73719318	C/A-/+	296bp	207,89,63,26bp
	AA+/+		207,63,26bp
	CC-/-		158,142 bp
rs73381662	CG-/+	300bp	158,143,99,43bp
	GG+/+		158,99,43bp
	AA-/-		298,52bp
rs73381650	AG-/+	350bp	298,201,97,52bp
	GG+/+		201,97,52bp

Reaction mixture

S.No.	Chemical	Amount(50µl)
1.	1x Frankfurt buffer	45µl
2.	Fw primer	1µl
3.	Rw primer	1 µl
4.	taq polymerase	0.5 µl
5	DNA sample	2.5ul

(i) rs73719353-Sfc1

S.No.	Chemical	Amount(20µl)
1.	BSA	1µl
2.	PCR Product	16µl
3.	10x buffer	2µl
4.	Enzyme	1 µl

Product Incubate at 37°C for overnight

⇩

Confirm on 3% agarose gel

(ii) rs73719341 -BccI

S.No.	Chemical	Amount(20µl)
1.	BSA	0.5µl
2.	PCR Product	17µl
3.	10x buffer	2µl
4.	Enzyme	0.5 µl

Product Incubate at 37°C for overnight

⇩

Confirm on 3% agarose gel

(iii) rs73719318-HpyCH4V

S.No.	Chemical	Amount(20µl)
1	PCR Product	17µl
2.	10x buffer	2µl
3	Enzyme	1 µl

Product Incubate at 37°C for overnight

⇩

Confirm on 3% agarose gel

(iv) rs73381662-ScrFI

S.No.	Chemical	Amount(20µl)
1	PCR Product	10µl
2.	10x buffer	2µl
3	Enzyme	1 µl
4	Sterile water	18ul

Product Incubate at 37°C for overnight

⇩

Confirm on 3% agarose gel

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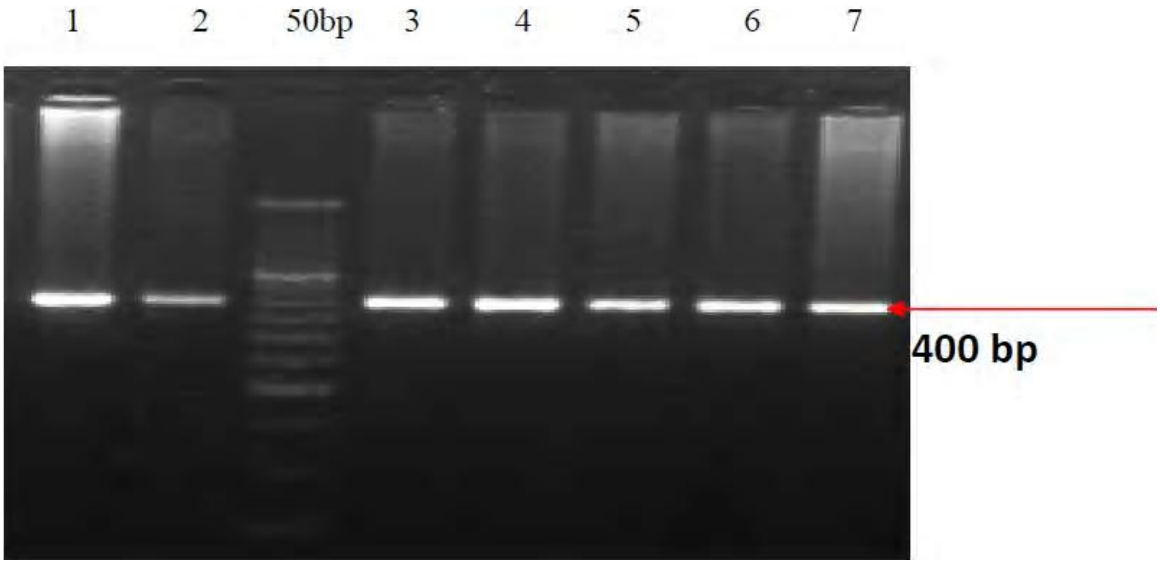

Figure 12 : Check gel of rs73719341

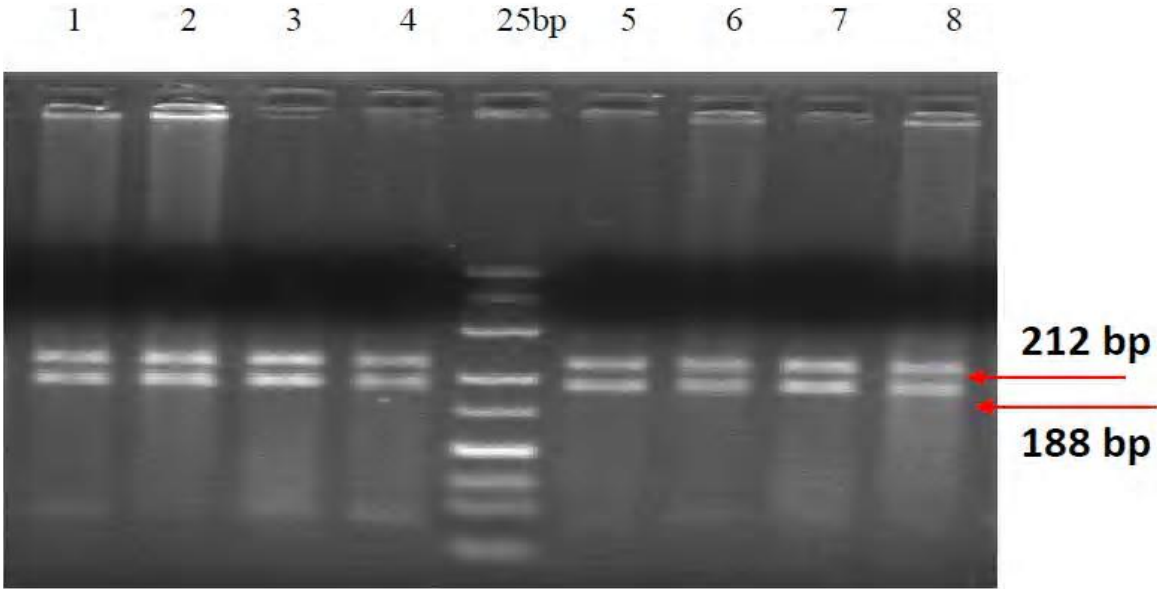

Figure 13 : Restriction digestion of rs73719341 with BccI

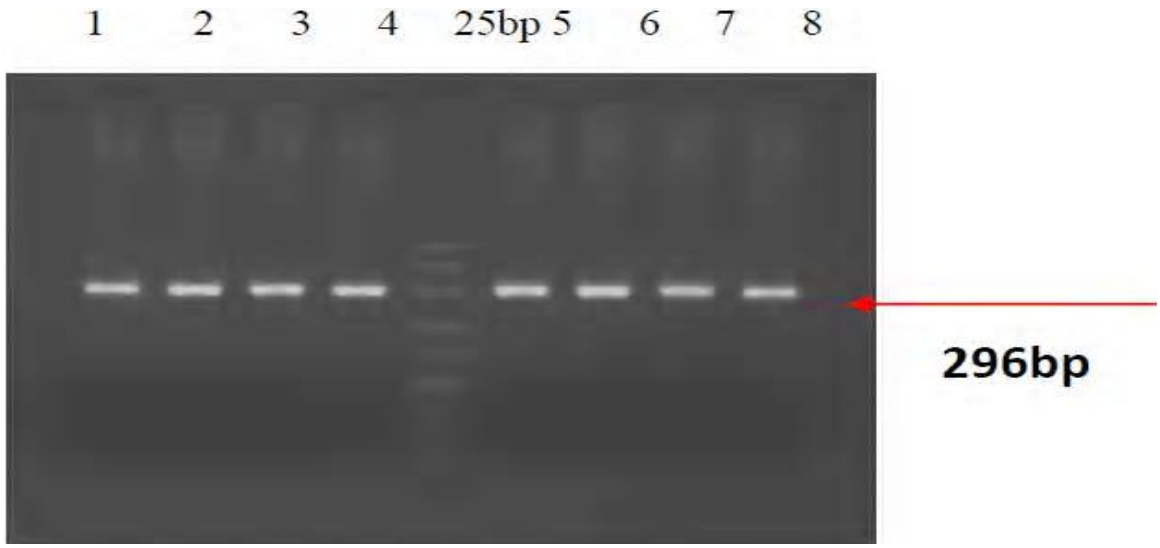

Figure 14 : Check gel of rs73719318

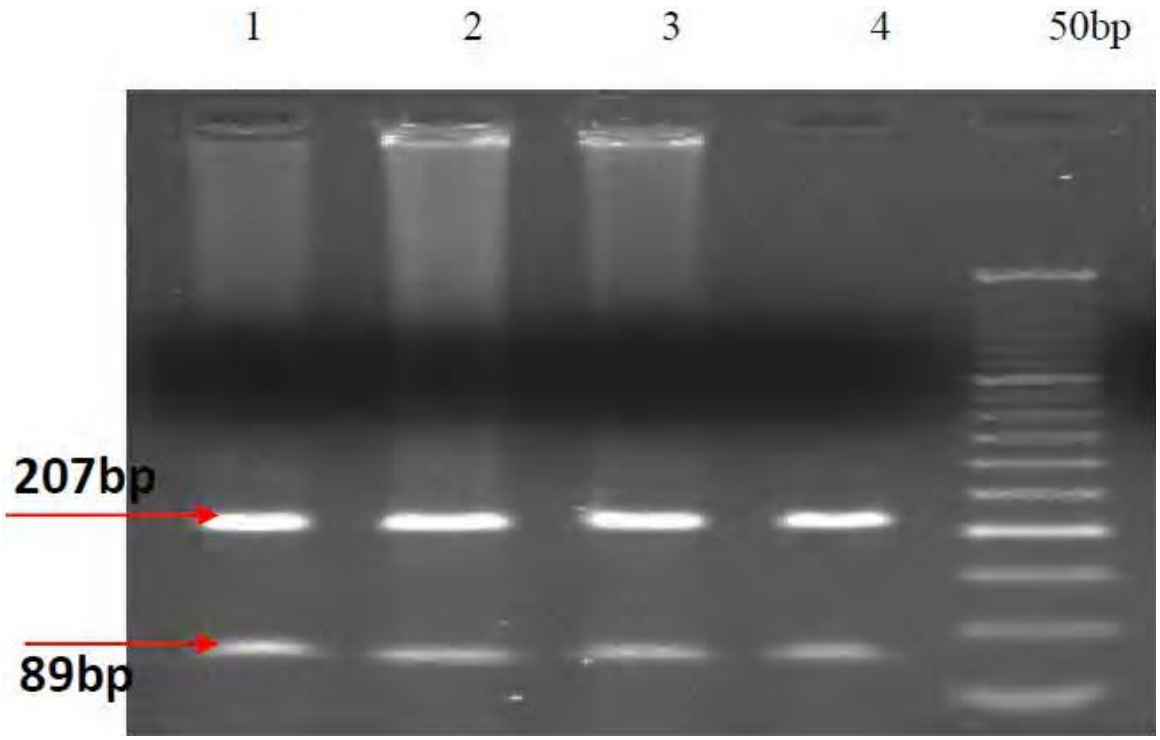

Figure 15 : Restriction digestion of rs73719318 with HpyCH4V

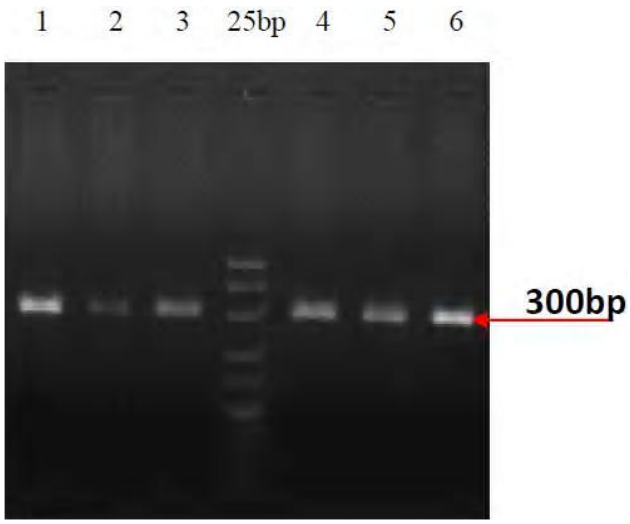

Figure 16 : Check gel of rs73381662

(v) rs73381650 -AflIII

S.No.	Chemical	Amount(20µl)
1	PCR Product	16.5µl
2.	10x buffer	2µl
3	Enzyme	1 µl
4	BSA	0.5

Product Incubate at 37°C for overnight

⇩

Confirm on 3% agarose gel

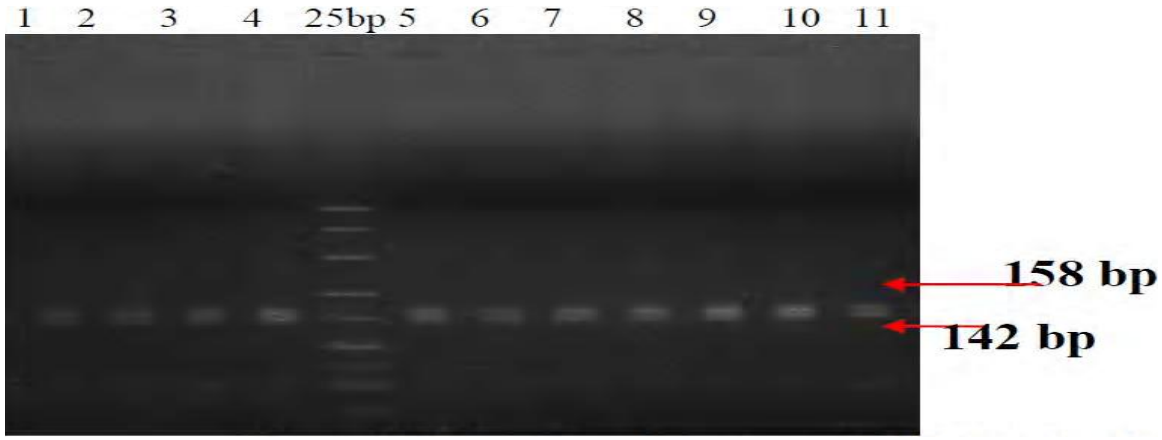

Figure 17 : Restriction digestions of rs73381662 with ScrFI

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6. Primer sequence and restriction enzyme of ANXA2 gene SNPs (sickle osteonecrosis) (Pandey et.al 2012)[10]

Gene	SNPs	Primer sequence	Restriction enzyme
ANXA2	rs7170178	5'-TTCACAGCAGTTCAAAATAC-3'F 5'- CTGGGTTTCCAGAGATGGAA-3'R	HpyCH4V
	rs73435133	5'-GAGTGCAAGGTGCTGAGGAT-3'F 5'- GATTTCAGACAGCCCTTGCA-3'R	DdeI
	rs73418020	5'- TCTGAGAGTGAAAGGTGCAC -3'F 5'-TCCCATCCCCTGAATCCCTG-3' R	HpyCH4III
	rs72746635	5'-CCTGACTCATTGTCACATCA-3'F 5'- AAGTGGGCTTTCCACTGCCC-3'R	DdeI
	rs73418025	5'-CTTCTCATCTTACTTTT-3'F 5'- AGGGAAGGATACAGAGGAGA-3'R	Sau 961

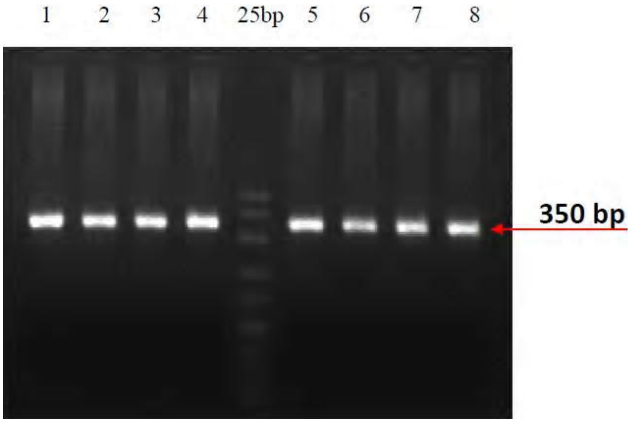

Figure 18 : Check gel of rs73381650

Restriction digestion

7. Thrombosis mutation

(i)Factor V Leiden[11]

Primer sequence

5'TGCCCACTGCTTAACAAGAAC3'Fw
5'TGTTATCACACTGGTGCTAA3'Rw

PCR –program for amplification

- Incubate at 95^{0}C for 00 :06:00
- Incubate at 95^{0}C for 00 :01:00
- Incubate at 59^0 C for 00 :00:30
- Incubate at 72^{0}C for 00 :01:00

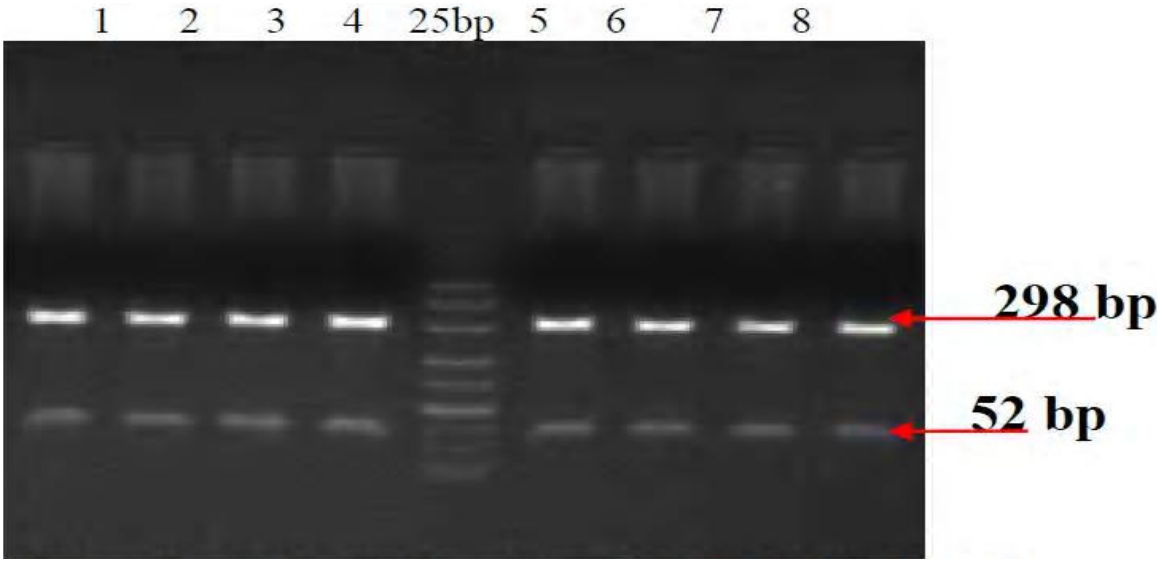

Figure 19 : Restriction digestion of rs73381650 with AflIII

Reaction mixture (common for all 5 SNPs)

S.No.	Chemical	Amount(50µl)
1.	1x Frankfurt buffer	45µl
2.	Fw primer	1µl
3.	Rw primer	1 µl
4.	Taq polymerase	0.5 µl
5	DNA sample	2.5ul

SNPs genotype of ANXA2 and restriction product size

SNPs	Genotype	Product size	
		Total product size	Restriction fregments product size
	GG-/-		338,106,72,34bp
rs7170178	GA-/+	550bp	338,195,143,106,72,34bp
	AA+/+		195,143,106,72,34bp
	GG+/+		185,110,39,16bp
rs73435133	GC+/-	350bp	295,185,110,39,16bp
	CC-/-		295,39,16bp
	GG-/-		240,110bp
rs73418020	G/A-/+	350bp	240,150,111,40bp
	AA+/+		200,110,40bp
	GG-/-		230,76,44bp
rs72746635	GA-/+	350bp	230,125,105,76,44bp
	AA+/+		125,105,76,44bp
	AA-/-		303,47bp
rs73418025	AG-/+	350bp	303,201,102,47bp
	GG+/+		201,102,47bp

- Cycle to step 2 for 35 more times.
- Incubate at 72^{0}C for 00 :10:00
- Incubate at 10^{0}C for 00 :15:00

ii) Methylenetetrahydrofolate reductase (MTHFR)[12]

Primer sequence

5'-CTTCTACCTGAAGAGCAAGTC-3' Fw
5'-CATGTCCACAGCATGGAG-3'Rw

(i) rs7170178- HpyCH4V

S.No.	Chemical	Amount(20µl)
1	PCR Product	17.5µl
2.	10x buffer	2µl
3	Enzyme	0.5 µl

Product Incubate at 37°C for overnight

⇩

Confirm on 3% agarose gel

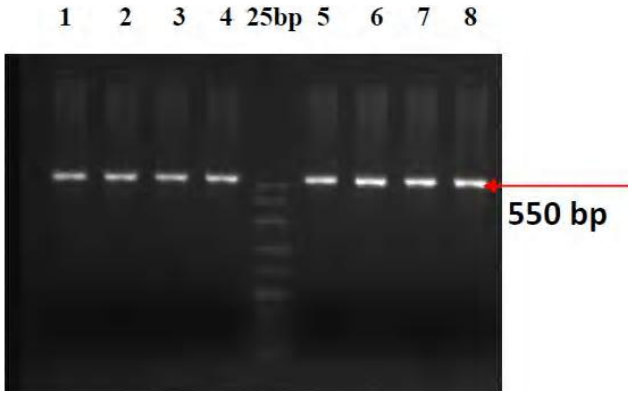

Figure 20 : Check gel of rs7170178

(ii) rs73435133 -DdeI

S.No.	Chemical	Amount(20µl)
1	PCR Product	17µl
2.	10x buffer	2.5µl
3	Enzyme	0.5 µl

Product Incubate at 37°C for overnight

⇩

Confirm on 3% agarose gel

(iii) rs73418020- HpyCH4III

S.No.	Chemical	Amount(20µl)
1	PCR Product	17µl
2.	10x buffer	2.5µl
3	Enzyme	0.5 µl

PCR –program for amplification

- Incubate at 95^{0}C for 00 :06:00

Full Paper

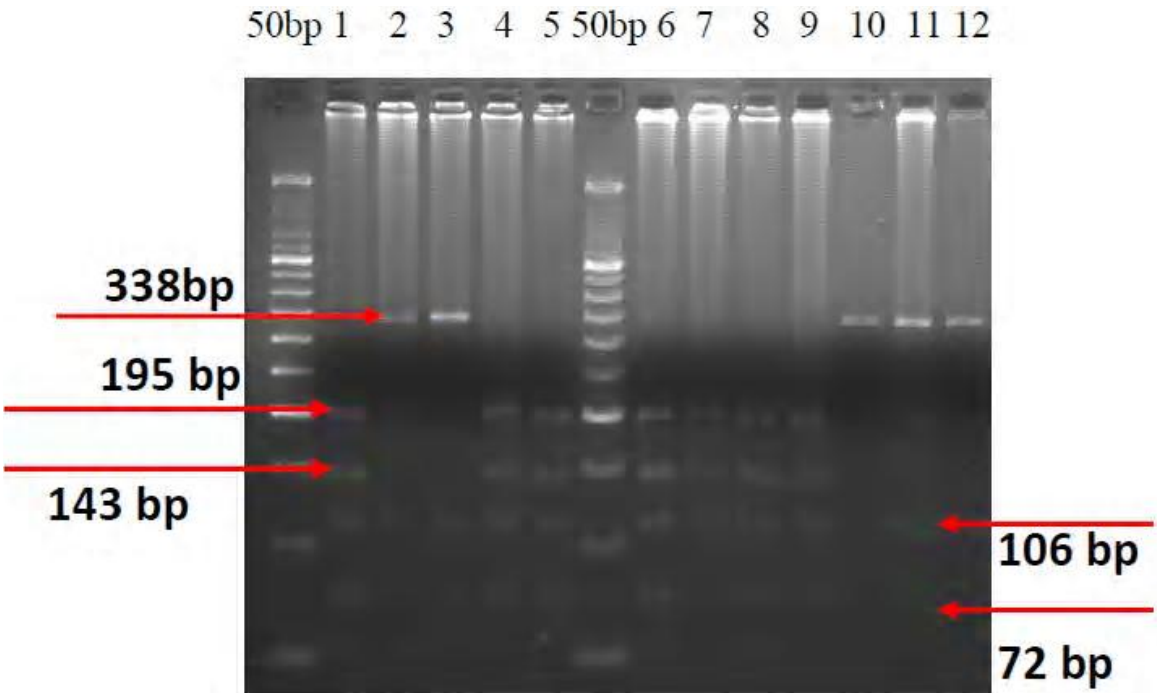

Figure 21 : Restriction Digestion of rs7170178 with HpyCH4V (Lane 1, 4, 5, 6, 7, 8, 9 mutant. 2, 3, 10 wild type. 11, 12 heterozygous)

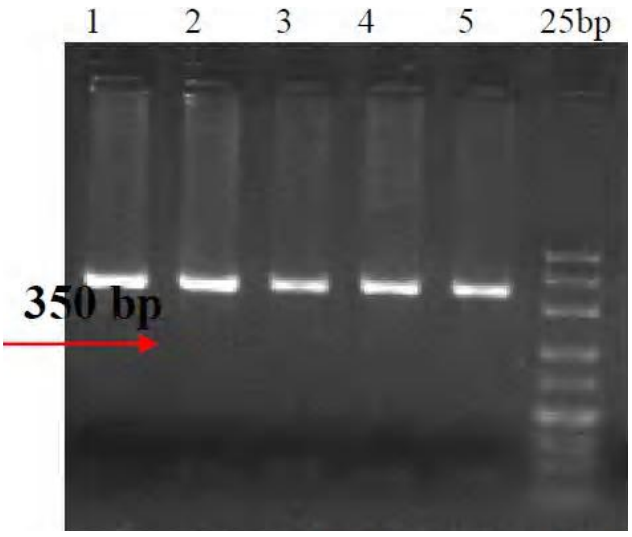

Figure 22 : Check gel of rs73435133

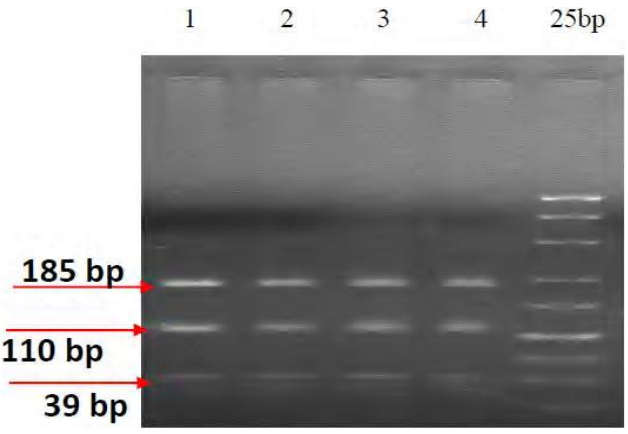

Figure 23 : Restriction digestion of rs73435133 with DdeI

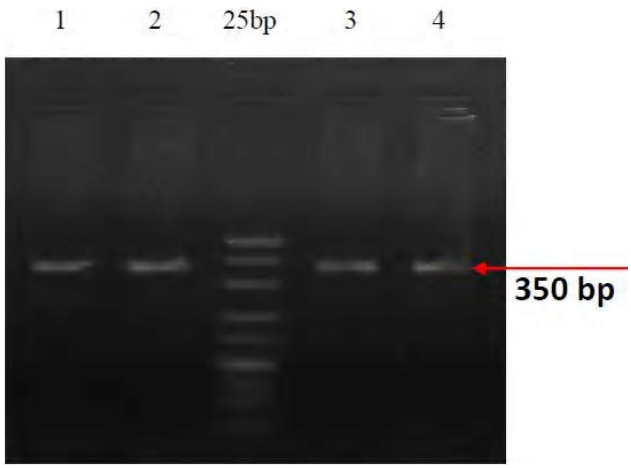

Figure 24 : Check gel of rs73418020

(iv) rs72746635 -DdeI

S.No.	Chemical	Amount(20µl)
1	PCR Product	17µl
2.	10x buffer	2.5µl
3	Enzyme	0.5 µl

Product Incubate at 37°C for overnight

⇩

Confirm on 3% agarose gel

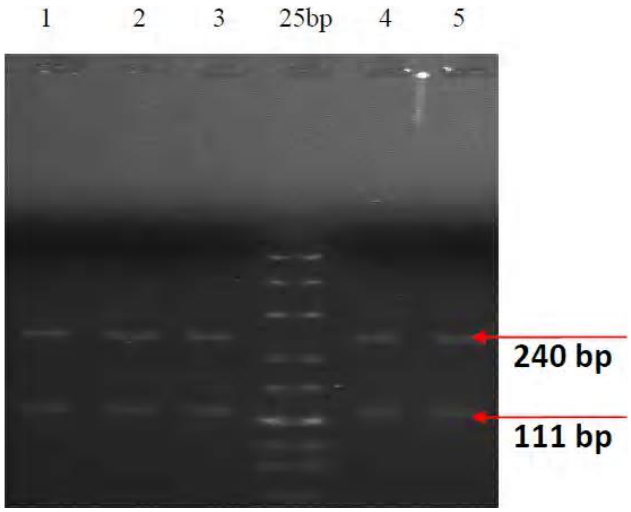

Figure 25 : Restriction digestion of rs73418020 with HpyCH4III

- Incubate at 95^{0}C for 00 :01:00
- Incubate at 59^0 C for 00 :00:30
- Incubate at 72^{0}C for 00 :01:00
- Cycle to step 2 for 35 more times.
- Incubate at 72^{0}C for 00 :10:00
- Incubate at 10^{0}C for 00 :15:00

(iii)Prothrombin mutation[13]

Primer sequence

5'TCTAGAAACAGTTGCCTGGC3'Fw
5'ATAGCACTGGGAGCATTGAAG3'Rw

PCR –program for amplification

- Incubate at 95^{0}C for 00 :06:00
- Incubate at 95^{0}C for 00 :01:00
- Incubate at 59^0 C for 00 :00:30

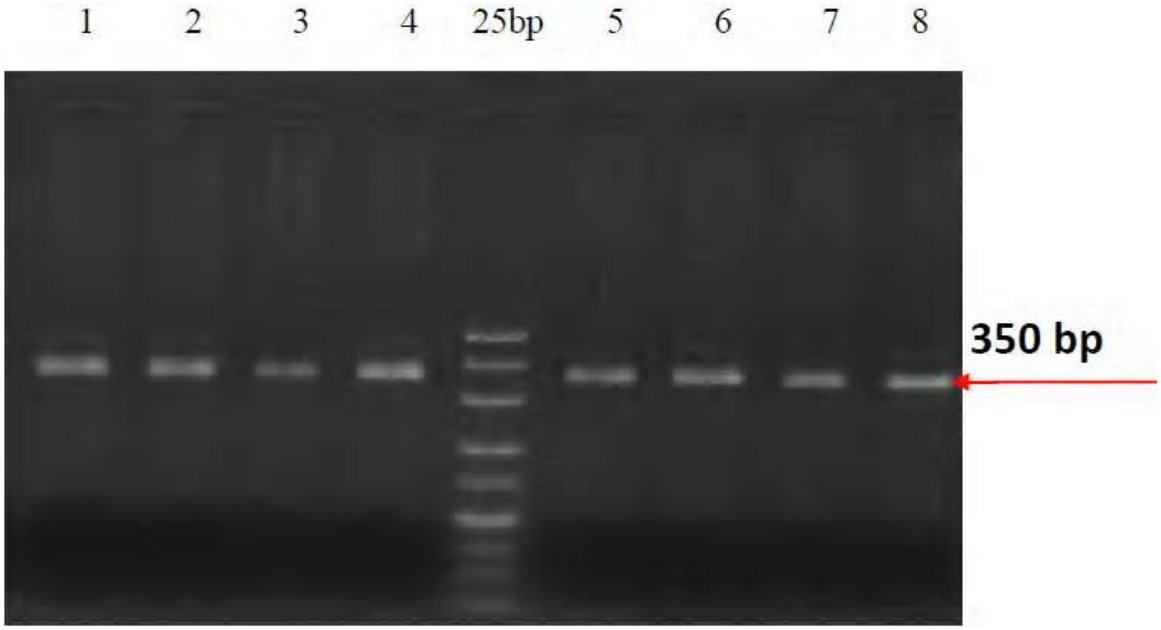

Figure 26 : Check gel of rs72746635

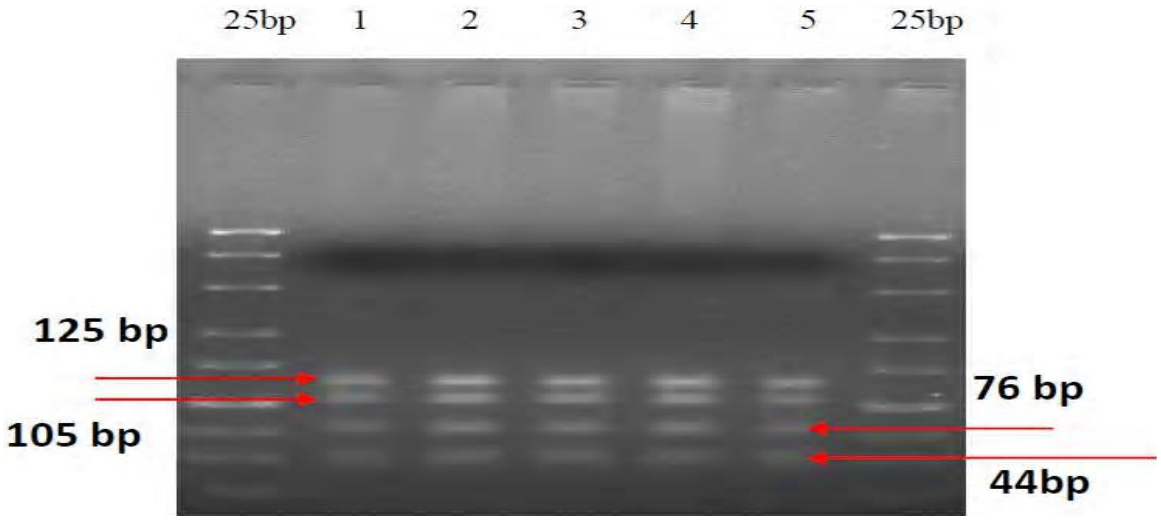

Figure 27 : Restriction digestion of rs72746635with DdeI

Full Paper

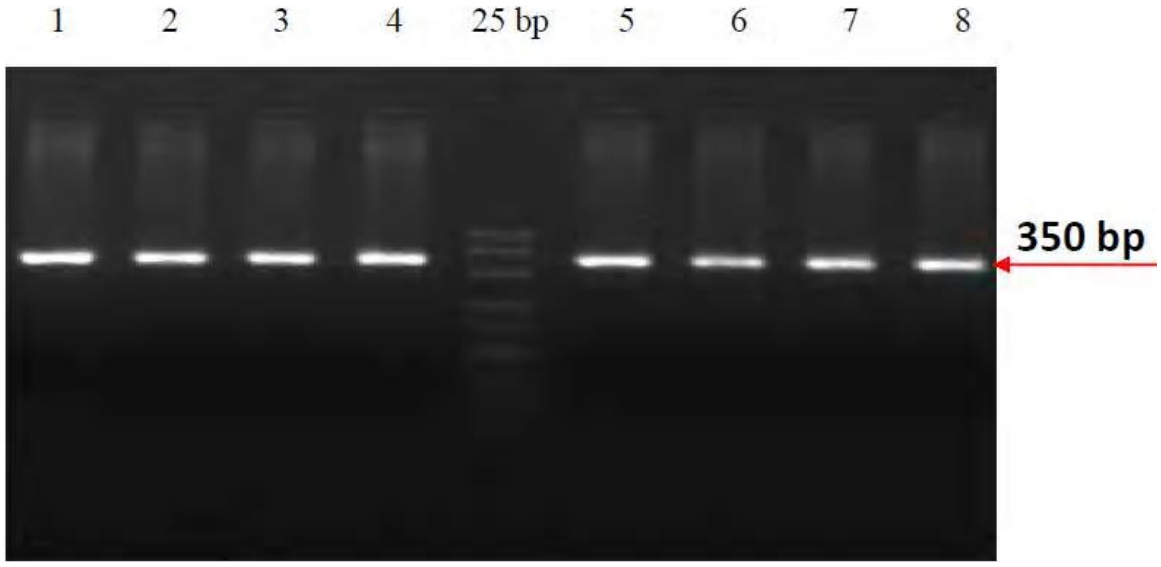

Figure 28 : Check gel of rs73418025

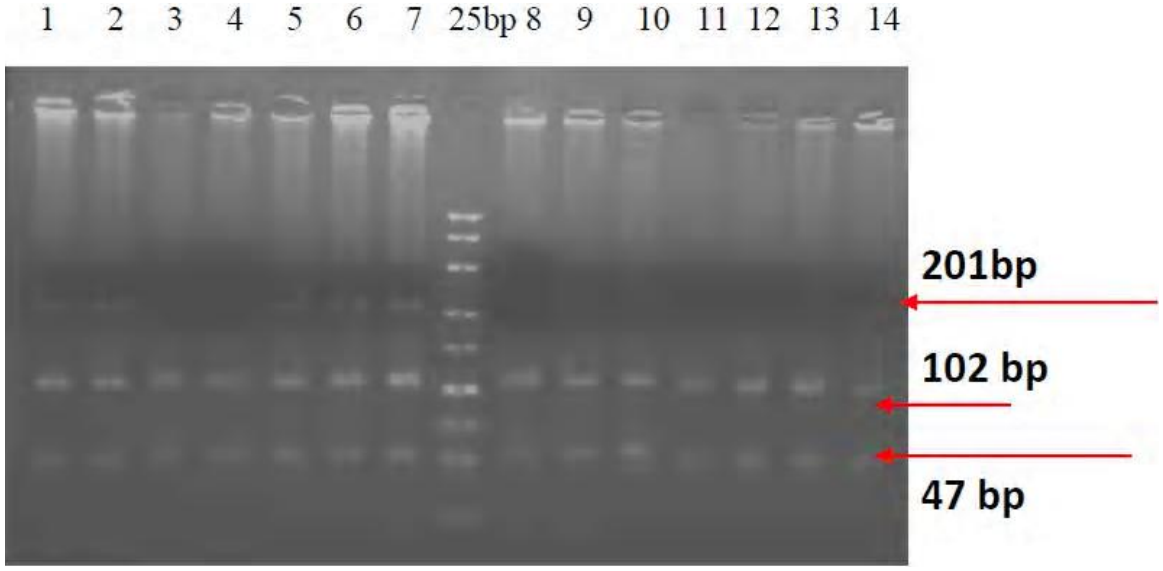

Figure 29 : Restriction digestion of rs73418025 with Sau 961

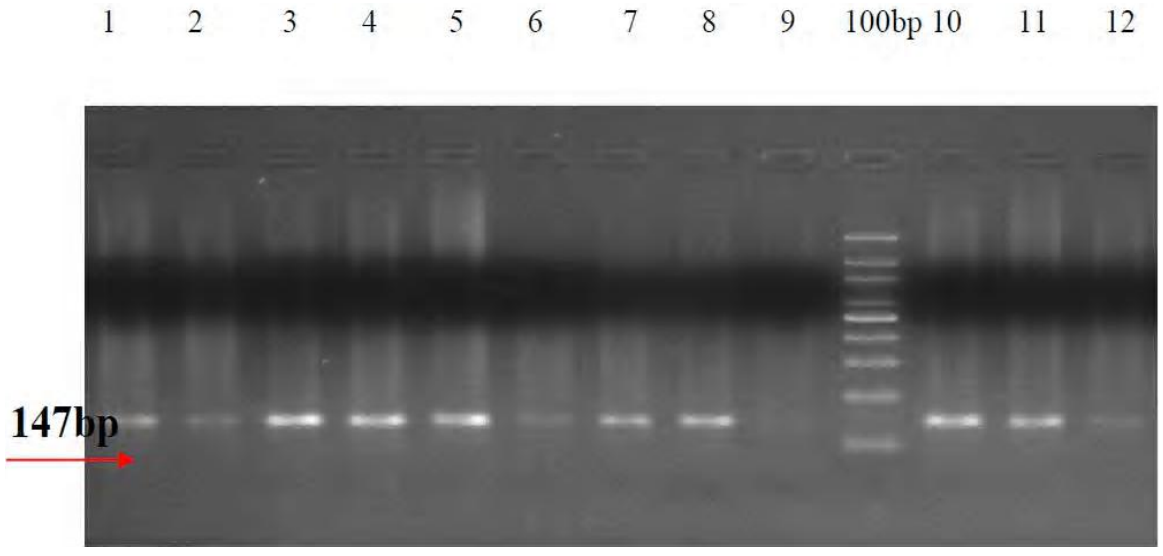

Figure 30 : Check gel for FV Leiden

(v) rs73418025- Sau 961

S.No.	Chemical	Amount(20µl)
1	PCR Product	17µl
2.	10x buffer	2µl
3	Enzyme	1 µl

Product Incubate at 37°C for overnight

⇩

Confirm on 3% agarose gel

Reaction mixture

S.No.	Chemical	Amount(26µl)
1.	1x Frankfurt buffer	22.5µl
2.	Fw primer	0.5µl (25pmol)
3.	Rw primer	0.5µl
4	Taq polymerase	0.5 µl
5	Sample DNA	2.0 µl

Restriction digestion for FV- Mnl-1

S. No.	Chemical	Amount(20µl)
1	PCR Product	17.5µl
2.	10x buffer	2µl
3	Enzyme	0.5 µl

Product Incubate at 37°C for overnight

⇩

Confirm on 3% agarose gel

Reaction mixture

S.No.	Chemical	Amount (26µl)
1.	1x Frankfurt buffer	22.5µl
2.	Fw primer	0.5µl
3.	Rw primer	0.5µl
4	Taq polymerase	0.5 µl
5	DNA Sample	2.0 µl

Restriction digestion for MTHFR -Hinf1

S.No.	Chemical	Amount(20µl)
1.	PCR Product	17.5µl
2.	10x buffer	2µl
3.	Enzyme	0.5 µl

Product Incubate at 37°C for overnight

⇩

Confirm on 3% agarose gel

- Incubate at 72^0C for 00 :01:00
- Cycle to step 2 for 35 more times.

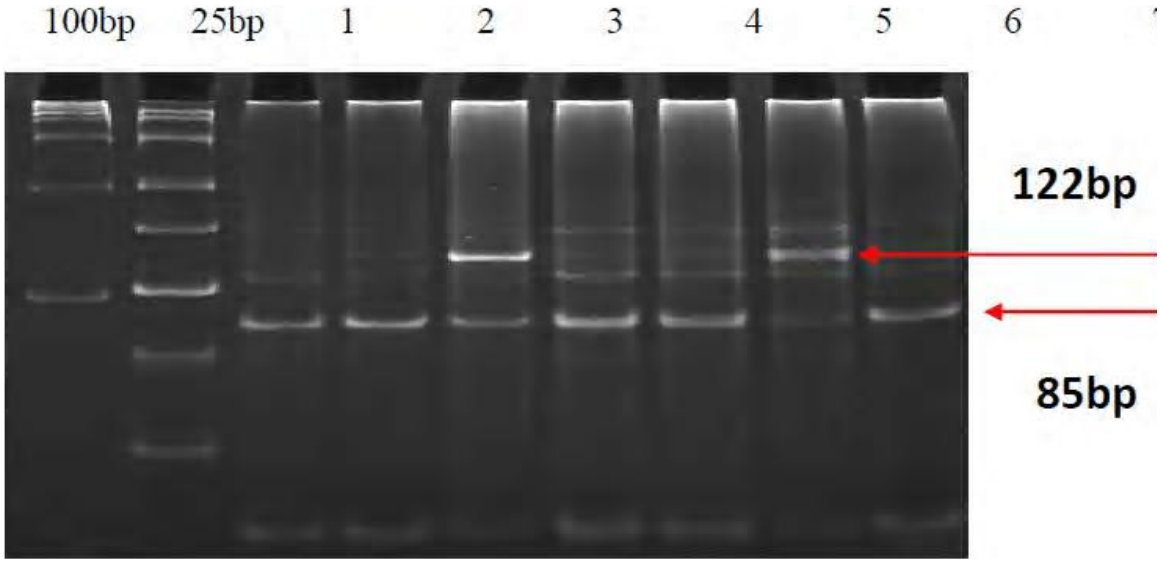

Figure 31 : Factor five Leiden mutation (lane 3, 6 heterozygous)

Full Paper

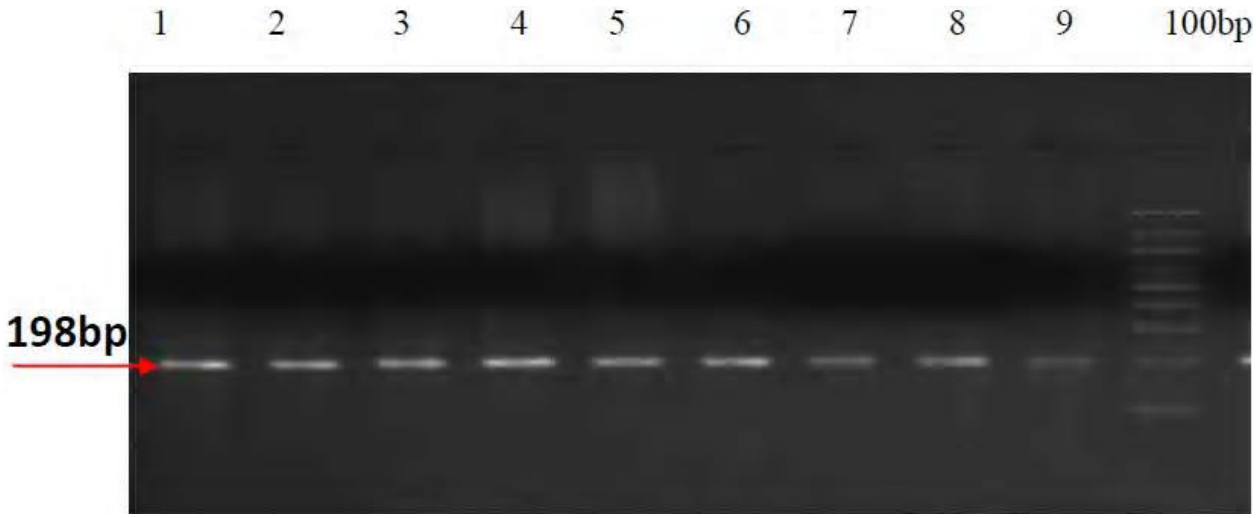

Figure 32 : MTHFR check gel

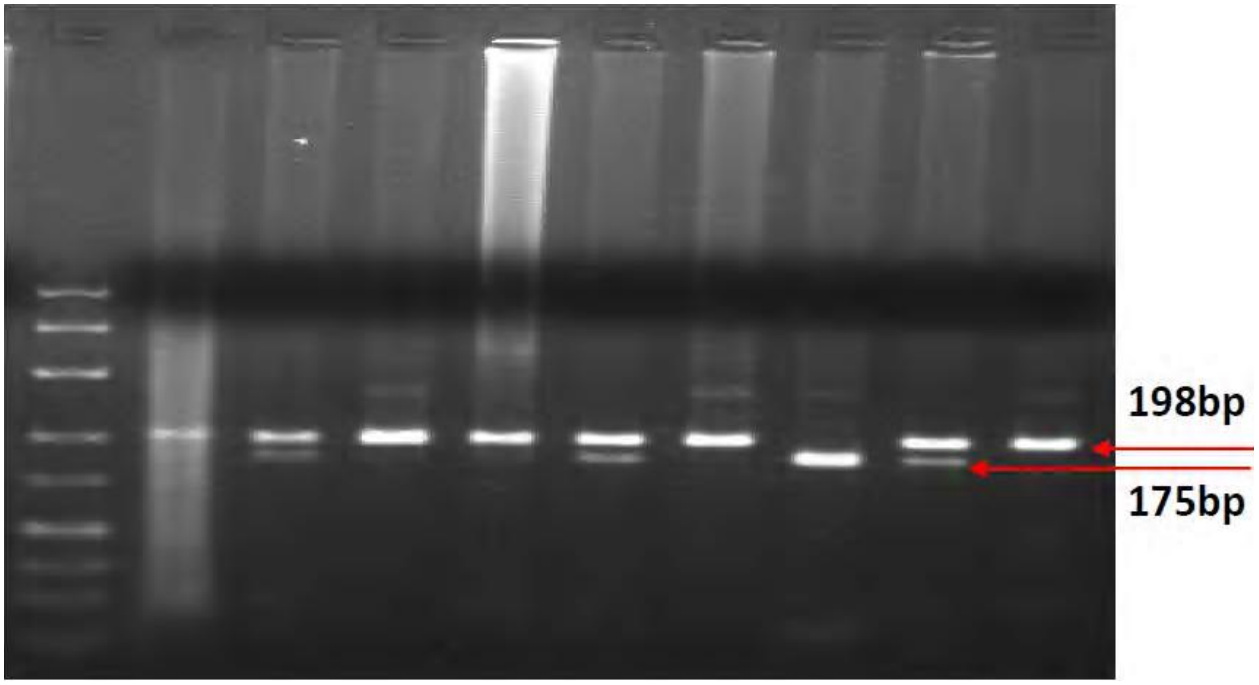

Figure 33: Restriction digestion for MTHFR (lane 2, 5, 8 are heterozygous and lane 7 is homozygous)

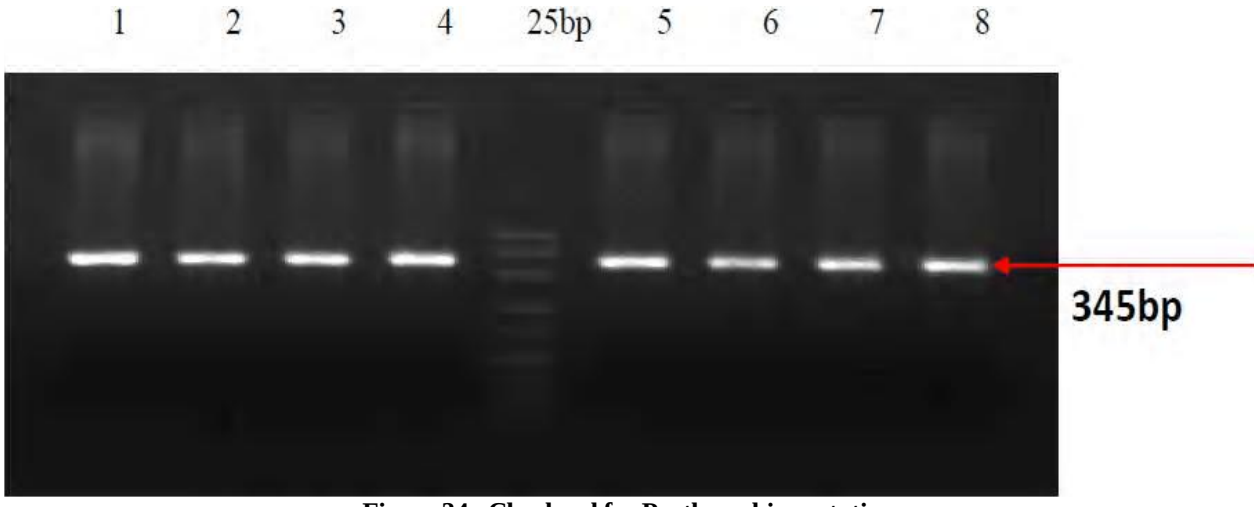

Figure 34 : Check gel for Prothrombin mutation

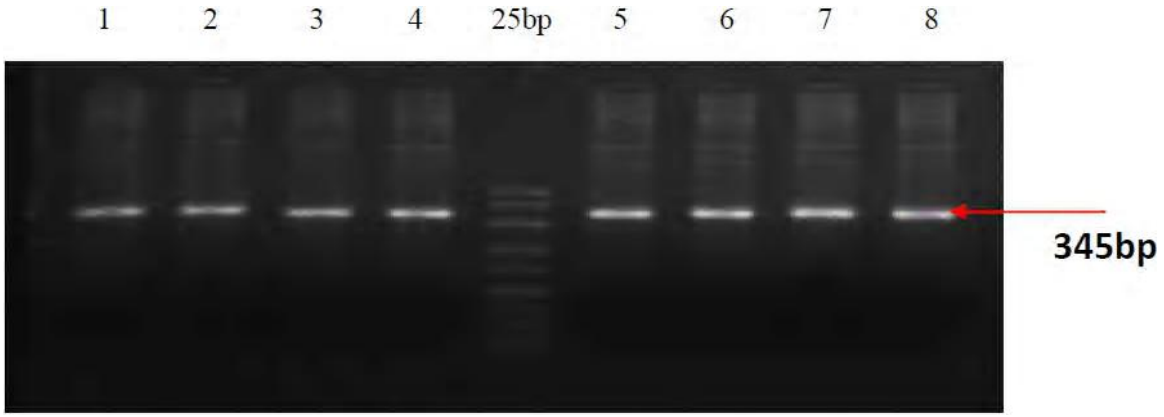

Figure 35 : Restriction digestion for prothrombin mutation (Not Digested)

Reaction mixture

S.No.	Chemical	Amount (26µl)
1.	1x Frankfurt buffer	22.5µl
2.	Fw primer	0.5µl
3.	Rw primer	0.5µl
4.	Taq polymerase	0.5 µl
5.	DNA sample	2.0 µl

Restriction digestion for prothrombin (PT) -Hind III

S.No.	Chemical	Amount (20µl)
1.	PCR Product	17.5µl
2.	10x buffer	2µl
3.	Enzyme	0.5 µl

Product Incubate at 37°C for overnight

⇩

Confirm on 3% agarose gel

- Incubate at 72^0C for 00 :10:00
- Incubate at 10^0C for 00 :15:00

CONCLUSION

Molecular diagnosis of disease based on PCR technology should be established in modern generalised hospital, pathological centre, and clinics. Method is rapid, cost effective and gold standard in detection of human disease and also simplest for persons working in molecular diagnostic and research laboratory. Technology is most useful in management of inherited disorder, genetic counselling and prenatal diagnosis of mutations.

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