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Review Article

MOLECULAR DETECTION OF INHERITED HAEMATOLOGICAL DISORDER

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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 counseling to DNA profiling for disease prevention, by performing entire genetic tests from hereditary diseases to infectious diseases from introduction of individualized medicines to prenatal diagnosis from non-invasive methods. Molecular Diagnosis of disease is very useful way to detect abnormalities in DNA level. These techniques are rapid, pragmatic advancement making them cost effective, accurate and setting gold standards. Various diseases i.e. Infectious (Viral, Bacterial, Fungal), Inherited and Malignancies can be detected in early stages to start medication and treatment that will reduce convalescence, monetary loss, mental stress and untimely demise. These molecular approaches should be made as an integral part of medical investigation to detect early to treat early approach in betterment of all over Health status of population at large.

KEYWORDS: PCR, Genotyping, DNA, Molecular Diagnosis, Early Detection of Diseases, Gold Standard, PPP.

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 attained gold standards status, 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 to avoid genetic diseases threat. But these investigations are not available to common mass people primarily because it's too costly and secondly due to huge population burden. Genetic diseases include thalassemia, sickle cell anaemia, hemophilia, thrombophilia, cystic fibrosis, Down syndrome etc. which take very large investment in its management, in some of them needs 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 Disorder**s- 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-II deficiency, 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 *CSF,* C. *pneumoniae* in CSF, Pulmonary Tuberculosis**,** Helicobacter pylori (Cryptosporidium spp.) Cryptosporidium, staphylococcus, *Mycoplasma pneumoniae, Neisseria*

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gonorrhoeae,Streptococcu.Mycobacterium, tuberculosis, Legionella Species, *Gardnerella vaginalis Clostridium difficile, Chlamydia trachomatis, Bartonella, Borrelia species.*

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 and other types of malignancies.

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.

EXPERIMENTAL METHODOLOGY:-

The worldwide accepted methodology for molecular detection of inherited hematological disorder is through the use of PCR technique which are now time tested, reliable & used by many researchers, pathologists & hematologists around the world and also recapitulated in our lab are well documented & described below:

Sample – Venous blood (1 ml)

DNA Extraction- Phenol chloroform Method and /or by Ready to Use DNA Extraction Kit Method.

Genotypic Detection

1. Genotypic detection of sickle cell by allele specific PCR: (Waterfall et al. 2001)[1]

Primer sequence

WT-AS (5′-ATG GTG CAC CTG ACT CCT GA-3′) WT- Fw control
CP517 (5′-CCC CTT CCT ATG ACA TGA ACT-3′) Rw control
MUT-AS (5′-CAG TAA CGG CAG ACT TCT CCA-3′) Fw mutant
MUT-CP267 (5′-GGG TTT GAA GTC CAA CTC CTA-3′) Rw mutant

PCR program for amplification

•Incubate at 95^{0}C for 00 :02:00

•Incubate at 95^{0}C for 00 :00:30

•Incubate at 65^{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(28µl)
1.	1x Frankfurt buffer	20µl
2.	Fw primer	0.5µl
3.	Rw primer	0.5µl
4.	$MgCl_2$	1 µl
5.	DMSO	3 µl
6.	Taq polymerase	0.5 µl
7.	DNA Sample	2.5 µl

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

1A 1B 2A 2B 3A 3B 4A 4B 5A 5B 6A 6B 7A 7B 8A 8B 9A 9B 1Kb

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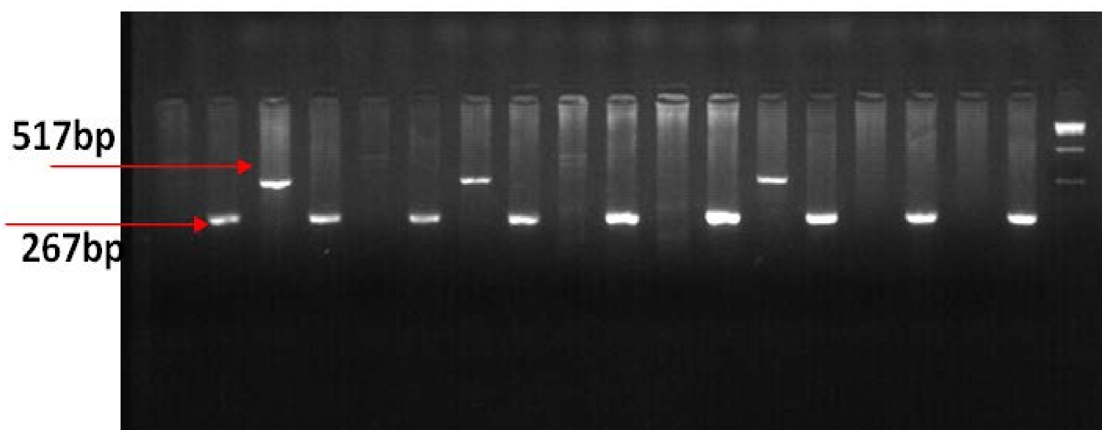

Fig.1: Gel picture of sickle cell gene (Lane 1,3,5,6,8,9 homozygous and 2,4,7 heterozygous)

2. Detection of alpha thalassemia by Gap- PCR

(I) - $\alpha^{3.7\ Kb}$ deletion: (Baysal et.al. 1994)[2]

Primer Sequence

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

PCR program for amplification

- 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.

A 1B 2A 2B 3A 3B 4A 4 B 1kb 100 bp

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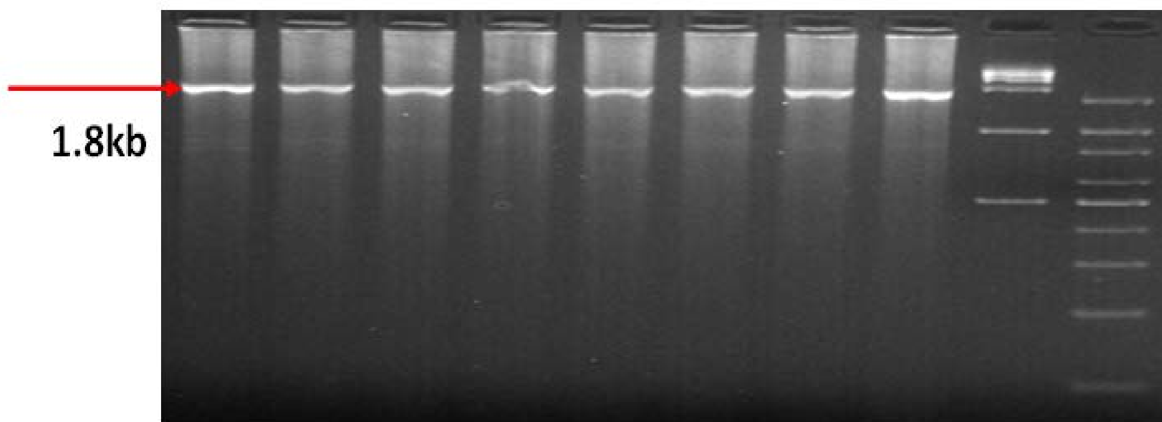

Fig. 2: Lane 1-4 Heterozygous (capital letters is a mutant set while small letters is normal set)

(ii) α $^{4.2}$kb deletion: (Baysal et.al.1994)[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

Reaction Mixture

S.No.	Chemical	Amount(28µl)
1.	1x Frankfurt buffer	20µ1
2.	Fw primer	0.5µ1
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.

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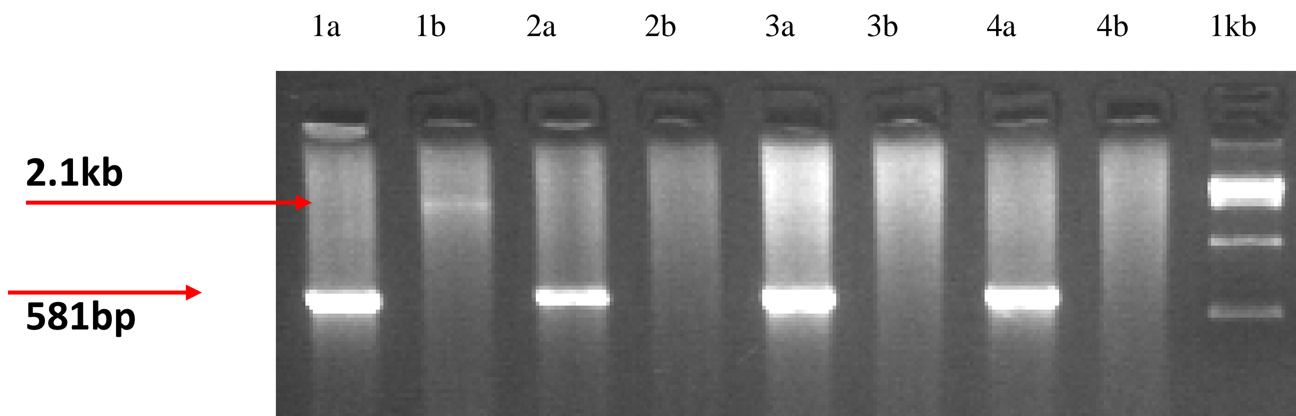

Fig. 3: α 4.2 kb deletion (Lane 1a 1b heterozygous)

(iii) --SEA délétion: (Chang et al.1991)[3]

Primer séquence

A. 5'-GCGATCTGGGCTCTGTGTTCT-3'

B. 5'-GTTCCCTGCCCCGACACG-3'

C. 5'-ACTGCAGCCTTGAACTCCTG-3'

PCR –program for amplification

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

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

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

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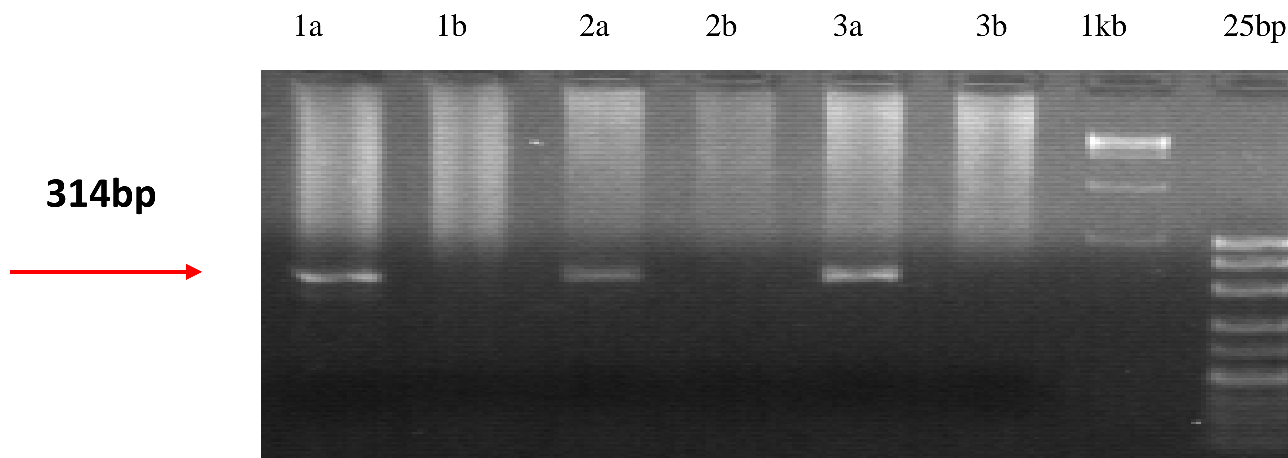

Fig. 4: South East Asian deletion (All normal)

(iv)--^{SA} deletion: (Shah et al 2003 and Tan et.al. 2001) [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

Reaction mixture

S.No.	Chemical	Amount (27µl)
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.	MgCl2	2.5 µl

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

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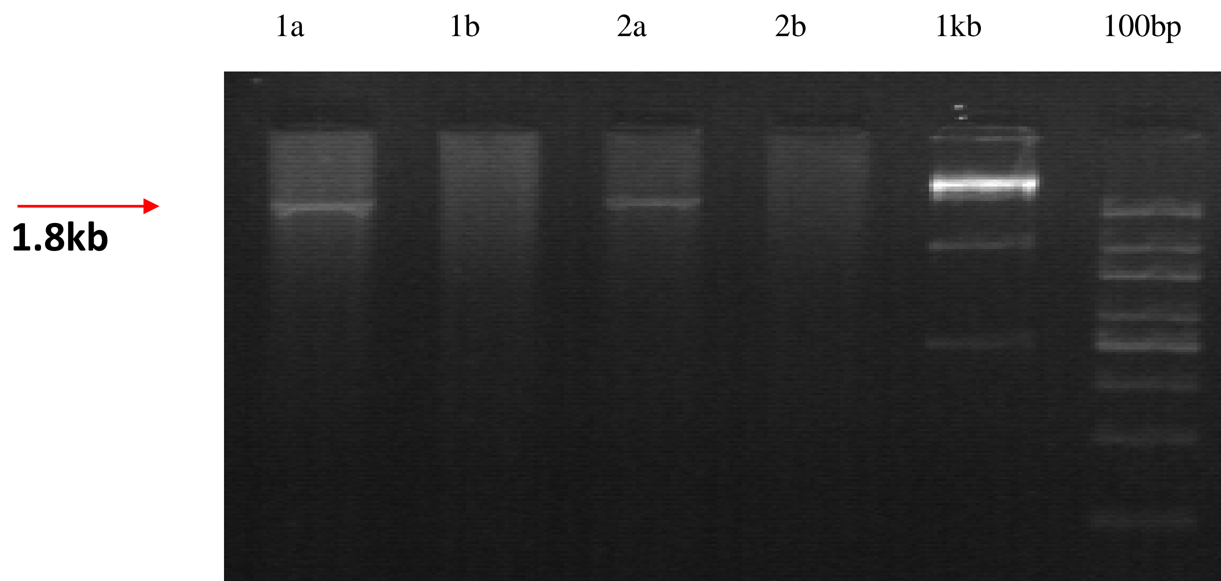

Fig. 5: South African deletion (All normal)

(v)Detection of α triplication (anti-3.7 del):-(*Smetanina* et.al. 1996) [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

Reaction mixture

S.No.	Chemical	Amount (28µl)
1.	1x Frankfurt buffer	20µ1
2.	Fw primer	0.5µ1
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

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

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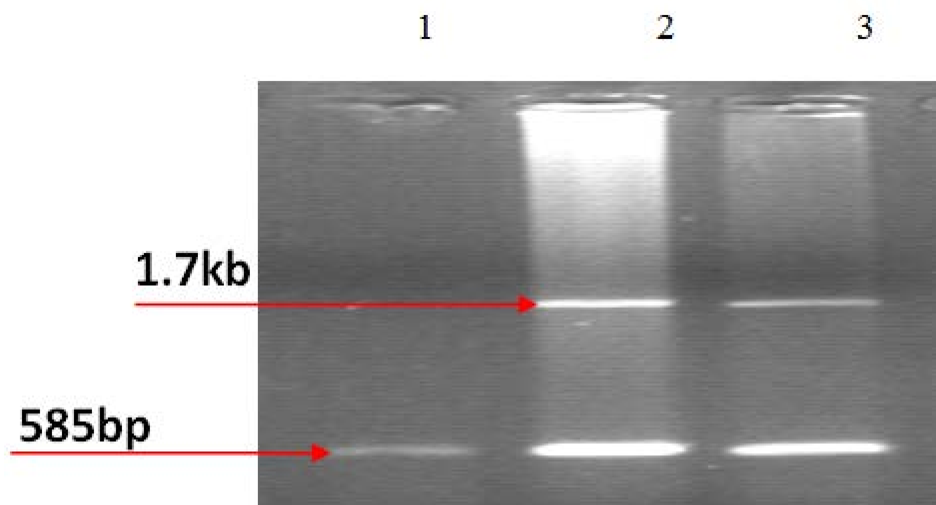

Fig.6: Alpha triplication (Lane 2,3 positive for anti 3.7 kb del.)

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

Beta thalassemia mutation screening: (Varawalla et al. 1991)[7]

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

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

- Incubate at 93^0 C for 00 :05:00
- Incubate at 93^0C for 00 :01:00
- Incubate at 66^0C for 00 :02:00
- Cycle to step 2 for 23 more times.
- Incubate at 93^0C for 00 :01:00
- Incubate at 66^0C for 00 :03:00
- Incubate at 72^0C for 00 :10:00
- Incubate at 10^0 C for 01 :00:00

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

1 2 3 100bp 4 5 6

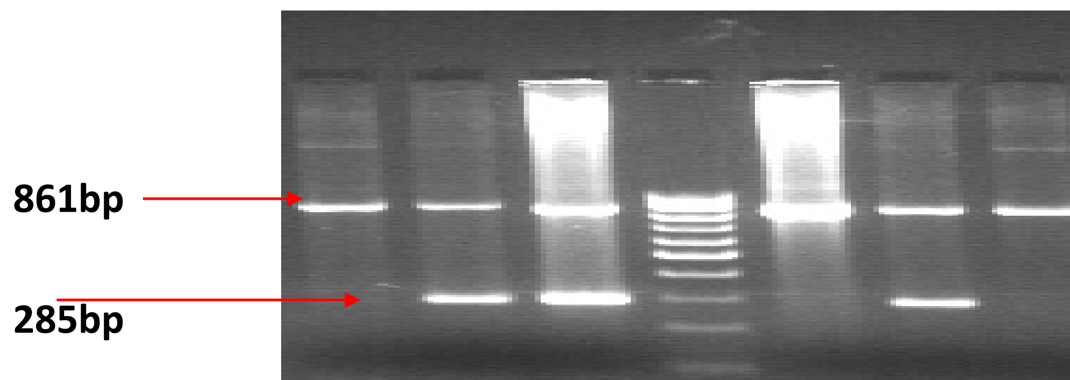

Fig.7: Beta thalassemia mutation (Lane 2, 3, 5 positive for IVS 1-5)

4. Detection of Xmn1 polymorphism:(Sutton et al. 1989)[8]

Primer sequence

Fw 5'-AACTGTTGCTTTATAGGATTTT-3'

Rw 5'-AGGAGCTTATTGATAACTCAGAC-3'

PCR program for amplification

•Incubate at 95^0C for 00 :05:00

•Incubate at 94 ^{0}C for 00 :01:00

•Incubate at 72^0C for 00 :01:00

•Cycle to step 2 for 30 more times.

•Incubate at 72^0C for 00 :05:00

•Incubate at 4^0C for 00 :10:00

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Reaction Mixture

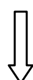
S.No.	Chemical	Amount(27µl)
1.	1x Frankfurt buffer	20µl
2.	Fw primer	1µl
3.	Rw primer	1 µl
4.	Taq polymerase	0.5 µl
5	DNA sample	2.5ul

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

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

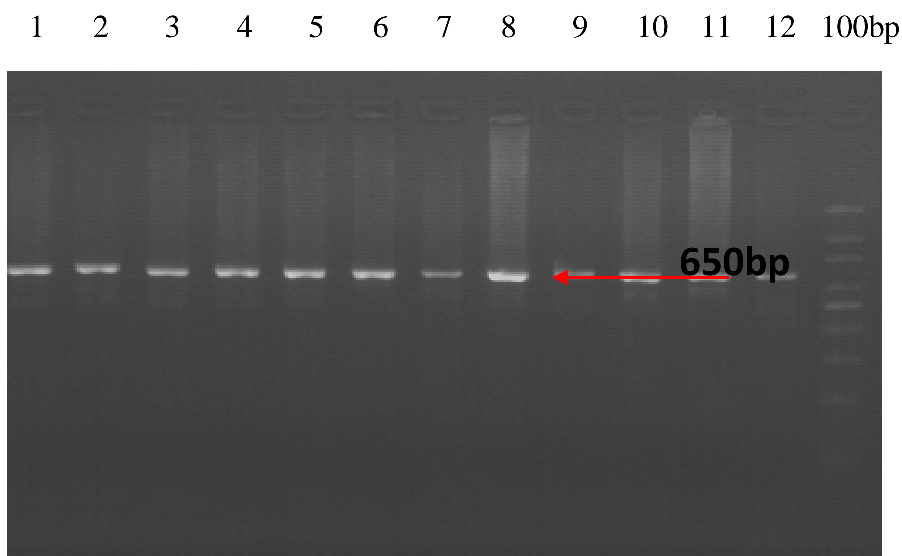


Fig. 8: Check gel for *XMN1*

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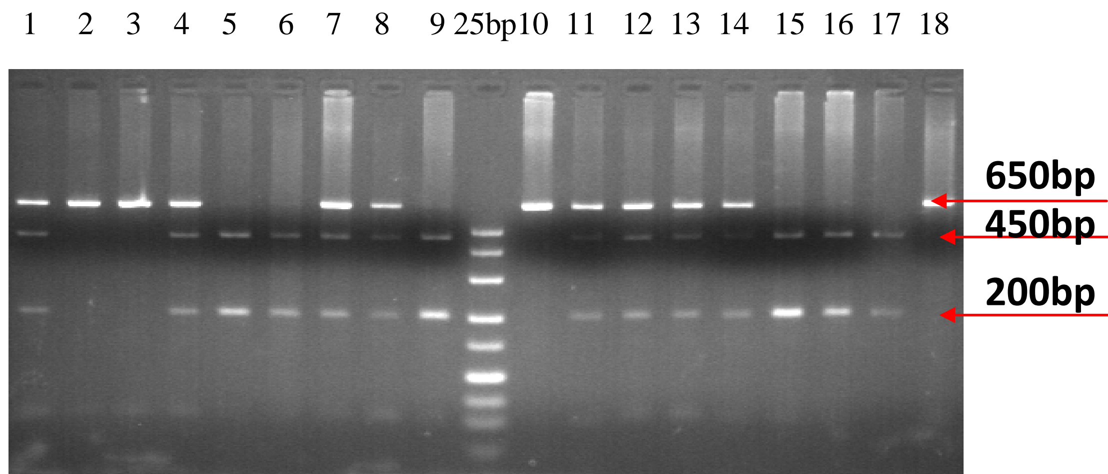

Fig. 9: Restriction digestion with Xmn-1 enzyme.

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	Sfc1
		5'-AGGCTCTGCTGAGCTCCTAC-3' R	
	rs73719341	5'-TGAACTTCCCATTCCCCTCT-3' F	BccI
		5' ATAAAATTAGCATTGATCCA 3' R	
	rs73719318	5'- CAGGT GCTGTGCAAC TTCTT -3'F	HpyCH4V
		5'-AGAGGGCACCATGGTTGCCT-3'R	
	rs73381662	5'- CTGAGATTCAATTAGGCCCA -3'F	ScrFI
		5'-TAAAGAACAGCAAAAGTCTG-3'R	
	rs73381650	5'-CACATAAAGATTGCTGCATT-3'F	AflIII
		5'- TAGTAATCCTAAAAATGGGA-3'R	

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

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SNPs genotype of BMP6 and restriction product size

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

Restriction Digestion

(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

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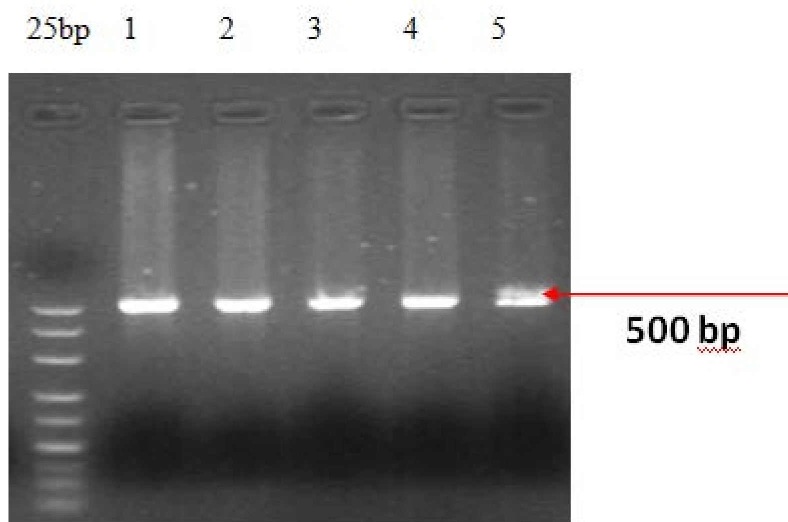


Fig. 10: Check gel of rs73719353

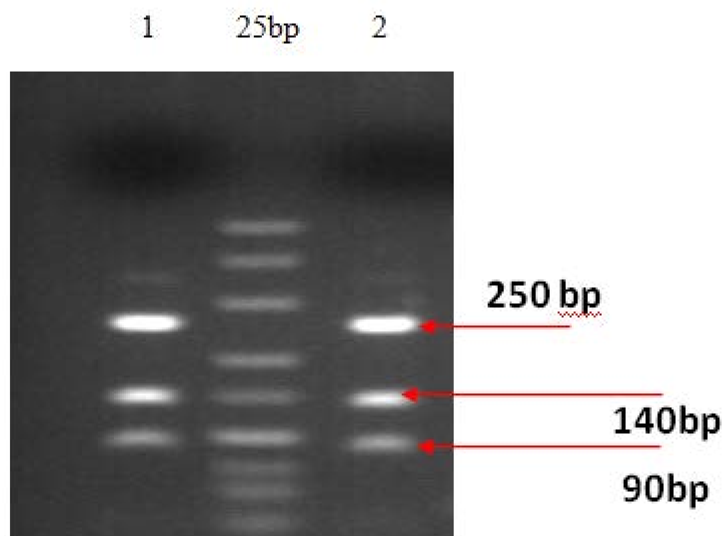
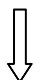


Fig. 11: Restriction digestion of rs73719353 with Sfc1

(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

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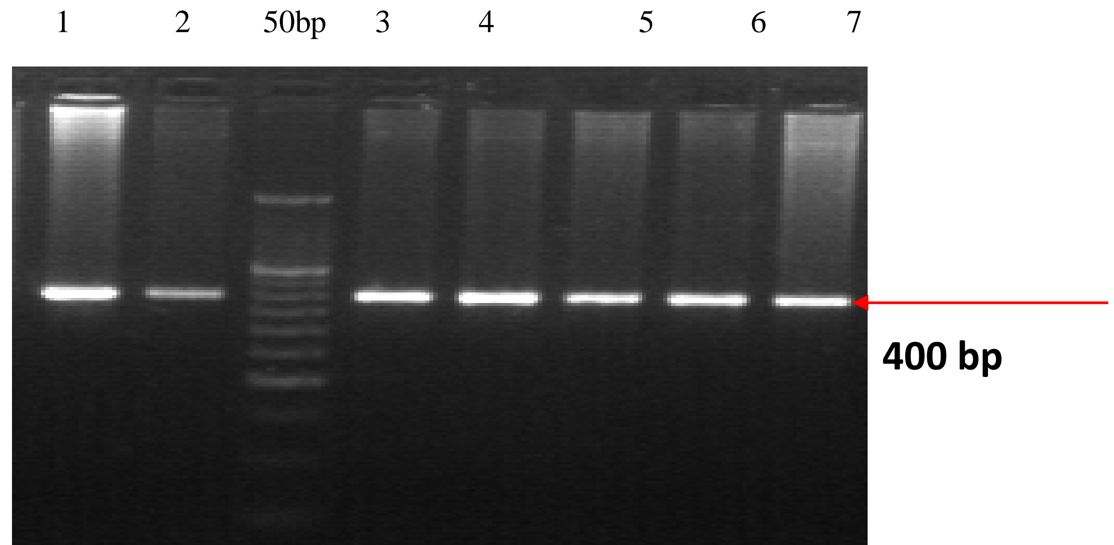


Fig.12: Check gel of rs73719341

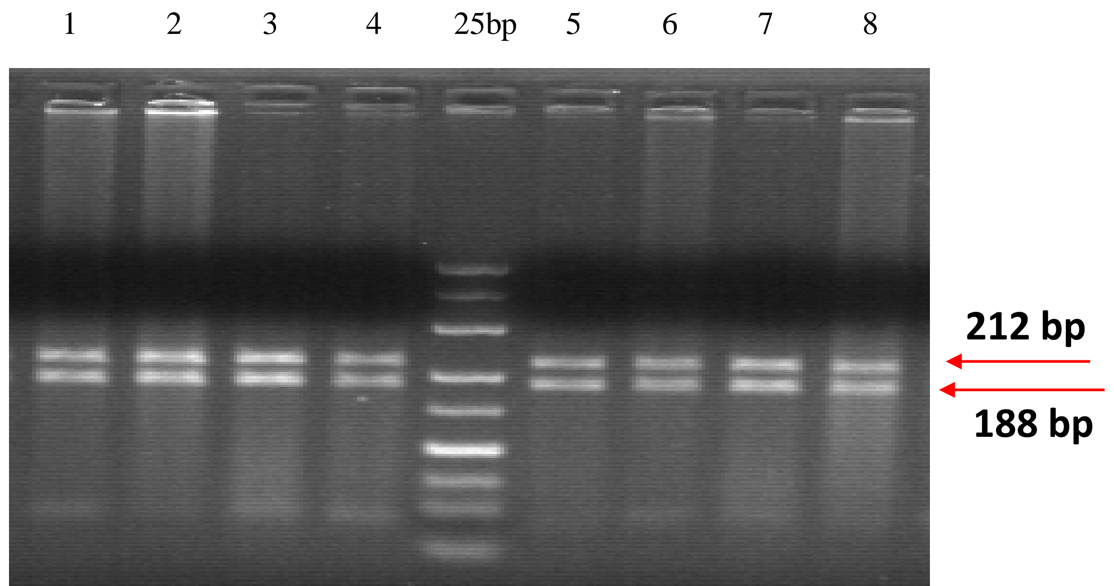
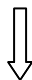


Fig.13: Restriction digestion of rs73719341 with BccI

(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

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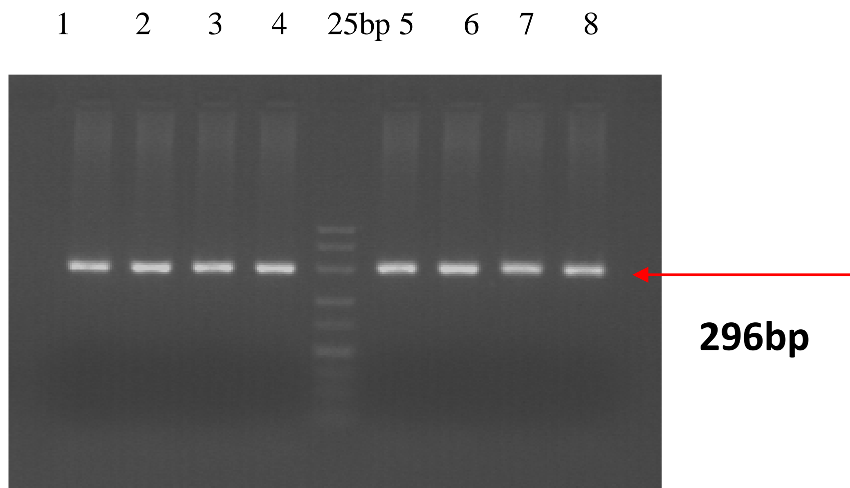


Fig.14: Check gel of rs73719318

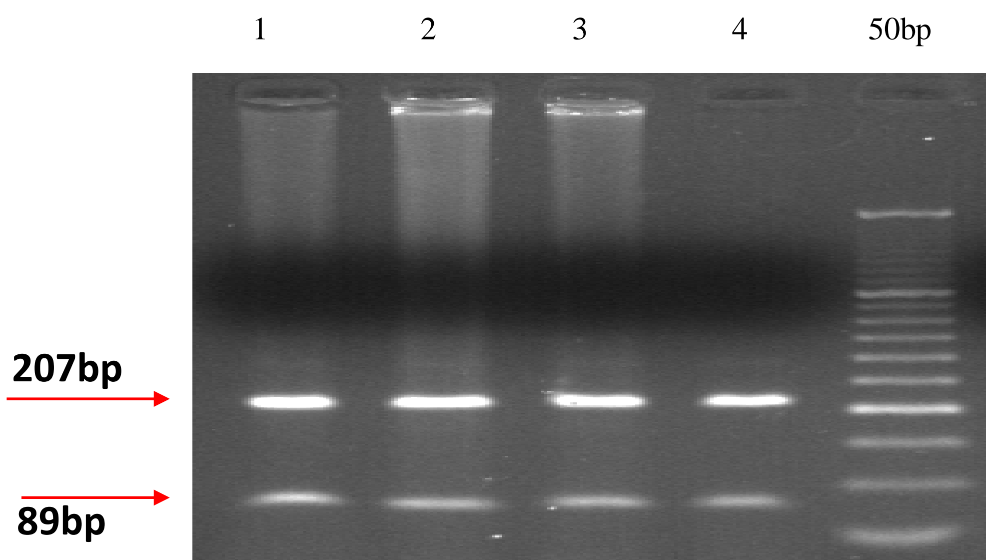
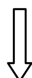


Fig.15: Restriction digestion of rs73719318 with HpyCH4V

(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% agrose gel

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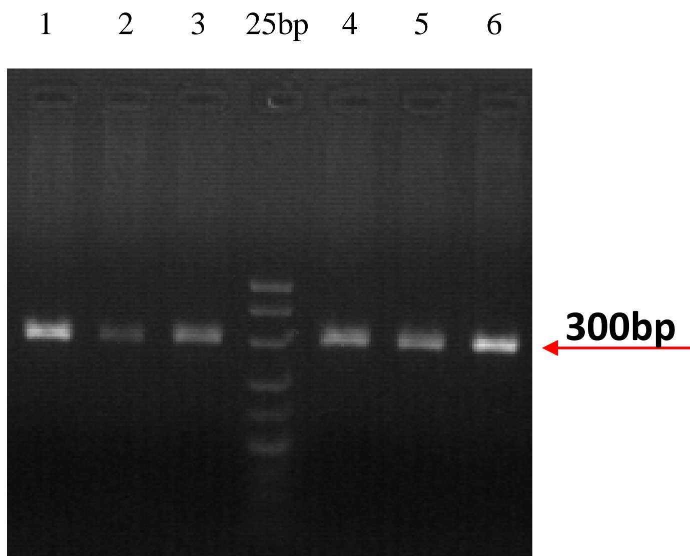

Fig.16: check gel of rs73381662

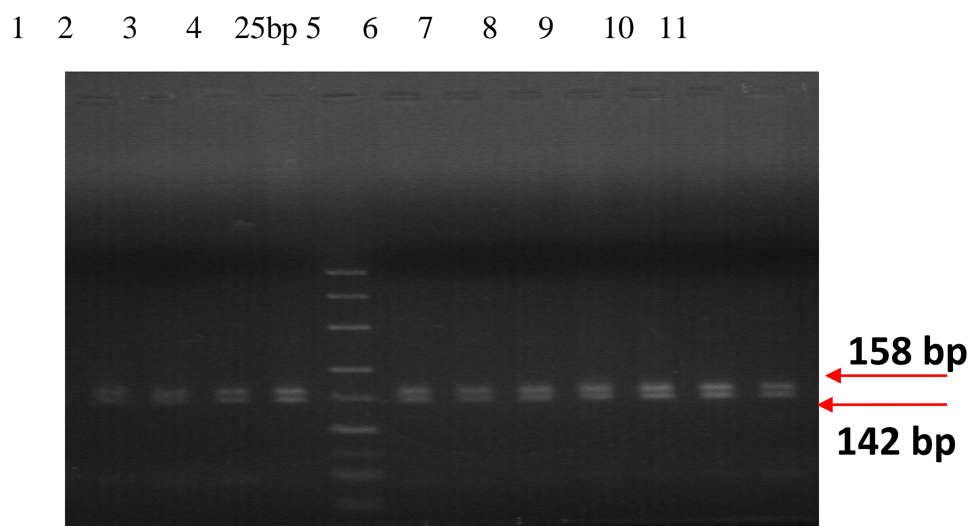

Fig. 17: Restriction digestions of rs73381662 with ScrFI

(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

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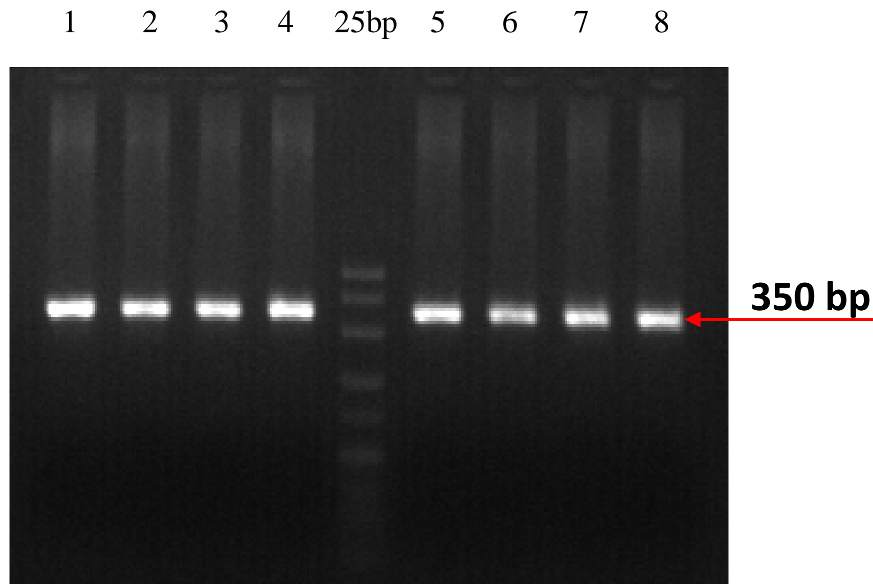

Fig.18: check gel of rs73381650

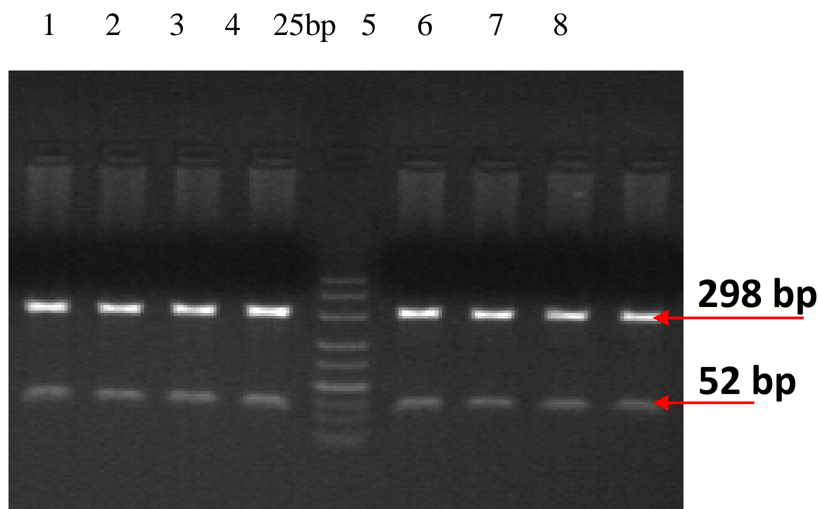

Fig. 19: Restriction digestion of rs73381650 with AflIII

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	HpyCH4V
		5'- CTGGGTTTCCAGAGATGGAA-3'R	
	rs73435133	5'-GAGTGCAAGGTGCTGAGGAT-3'F	DdeI
		5'- GATTTCAGACAGCCCTTGCA-3'R	

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	rs73418020	5'- TCTGAGAGTGAAAGGTGCAC -3'F	HpyCH4III
		5'-TCCCATCCCCTGAATCCCTG-3' R	
	rs72746635	5'-CCTGACTCATTGTCACATCA-3'F	DdeI
		5'- AAGTGGGCTTTCCACTGCCC-3'R	
	rs73418025	5'-CTTCTCATCTTACTTTT-3'F	Sau 961
		5'- AGGGAAGGATACAGAGGAGA-3'R	

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

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
rs7170178	GG-/-	550bp	338,106,72,34bp
	GA-/+		338,195,143,106,72,34bp

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	AA+/+		195,143,106,72,34bp
rs73435133	GG+/+	350bp	185,110,39,16bp
	GC+/-		295,185,110,39,16bp
	CC-/-		295,39,16bp
rs73418020	GG-/-	350bp	240,110bp
	G/A-/+		240,150,111,40bp
	AA+/+		200,110,40bp
rs72746635	GG-/-	350bp	230,76,44bp
	GA-/+		230,125,105,76,44bp
	AA+/+		125,105,76,44bp
rs73418025	AA-/-	350bp	303,47bp
	AG-/+		303,201,102,47bp
	GG+/+		201,102,47bp

Restriction digestion

(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

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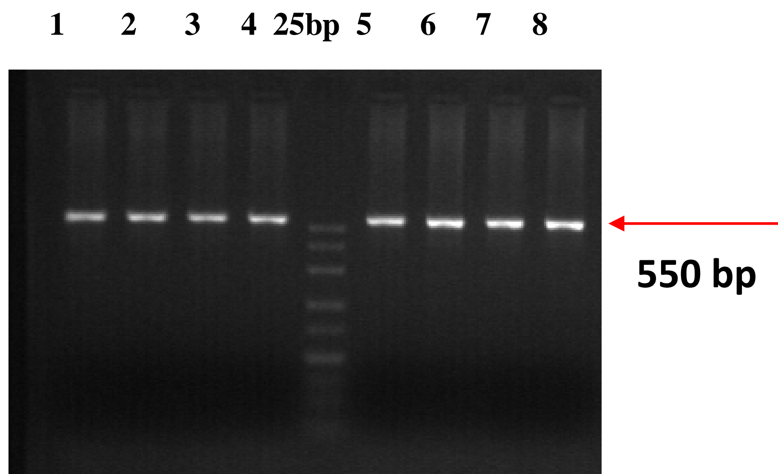

Fig. 20: Check gel of rs7170178

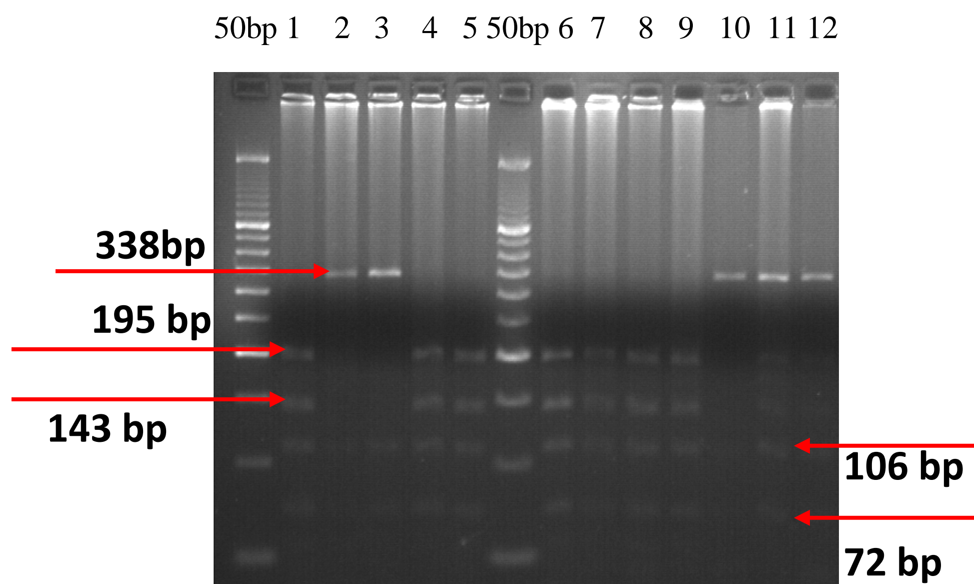

Fig. 21: Restriction Digestion of rs7170178 with HpyCH4V (Lane 1, 4, 5, 6, 7, 8, 9 mutant. 2, 3, 10 wild type. 11, 12 heterozygous)

(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

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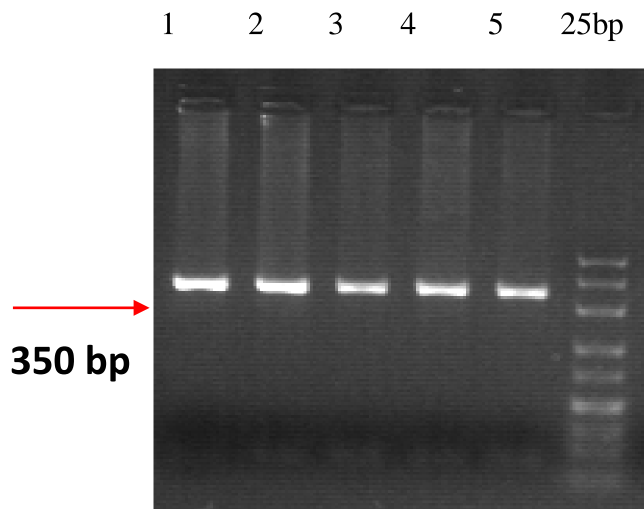

Fig. 22: Check gel of rs73435133

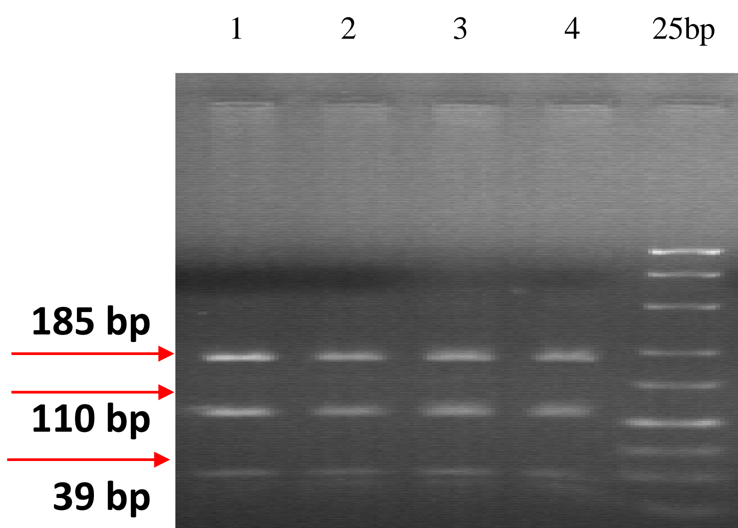

Fig. 23: Restriction digestion of rs73435133 with DdeI

(iii) **rs73418020- HpyCH4III**

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

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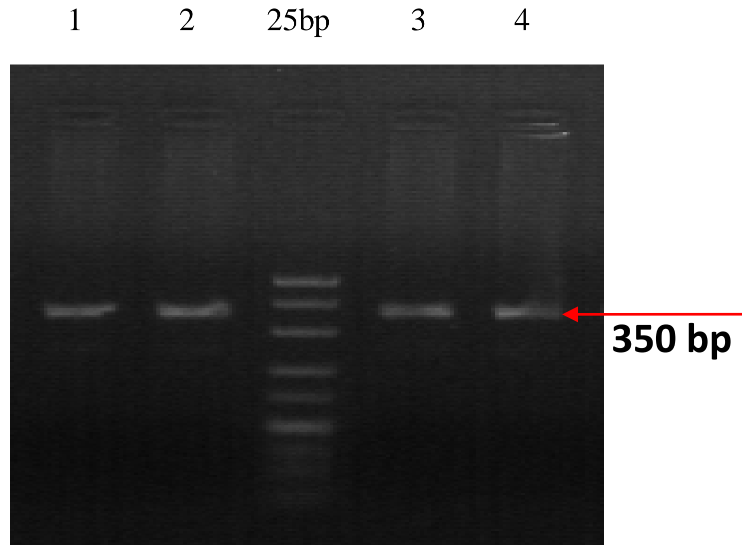

Fig. 24: Check gel of rs73418020

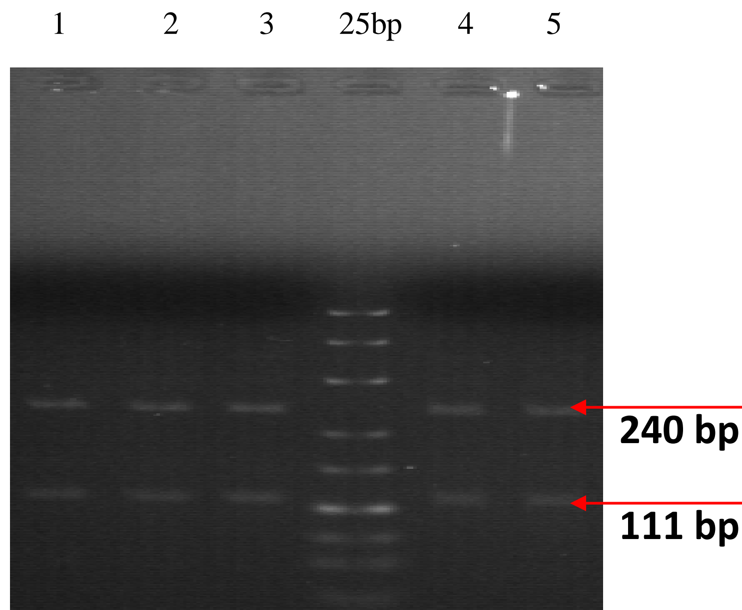

Fig. 25: Restriction digestion of rs73418020 with HpyCH4III

(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

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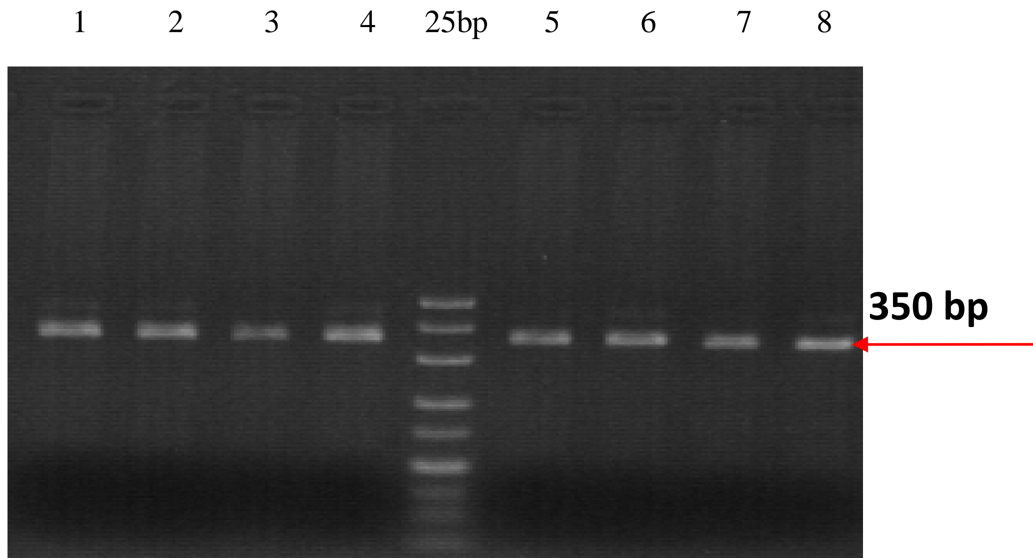

Fig. 26: Check gel of rs72746635

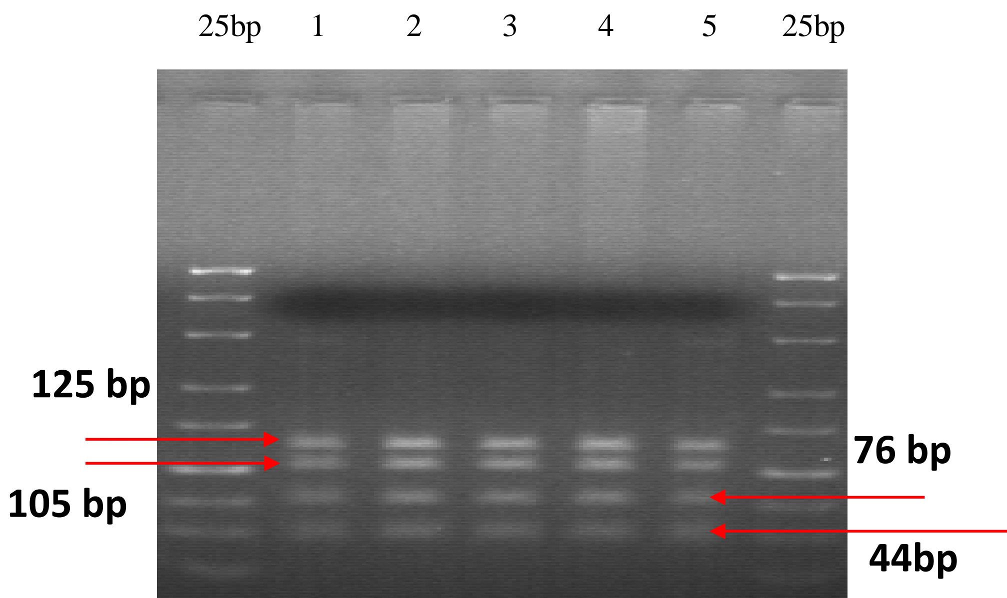

Fig. 27: Restriction digestion of rs72746635with DdeI

(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

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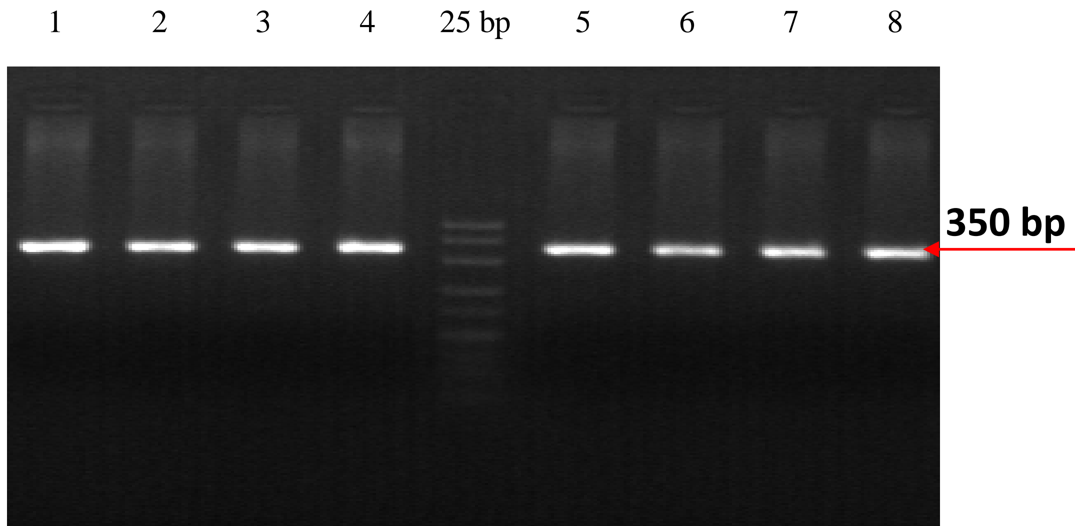

Fig. 28: Check gel of rs73418025

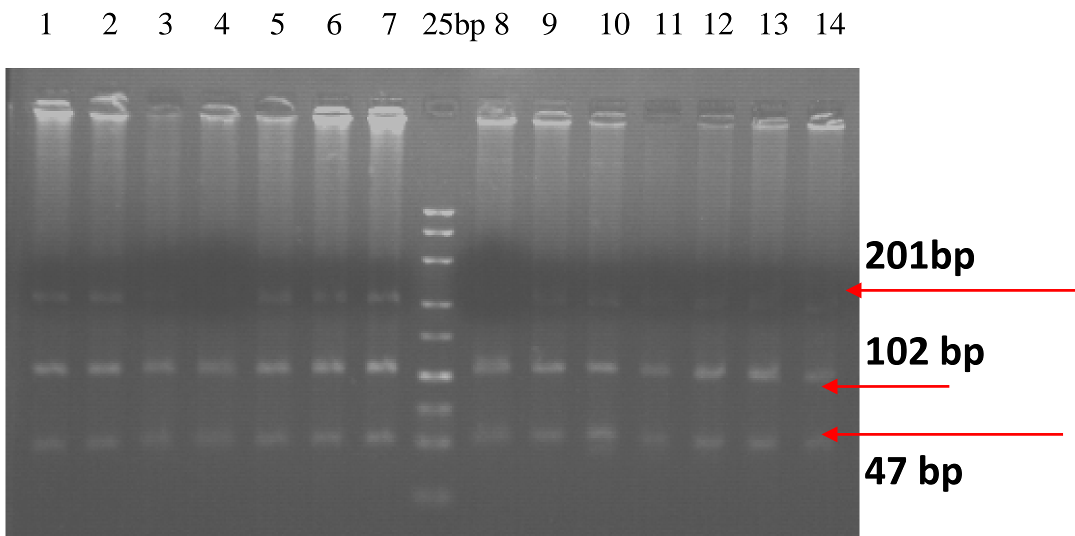

Fig. 29: Restriction digestion of rs73418025 with Sau 961

7. Thrombosis mutation

(i) Factor V Leiden: (Bertina et al.1994)[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
- 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

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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 10% Polyacylamide gel

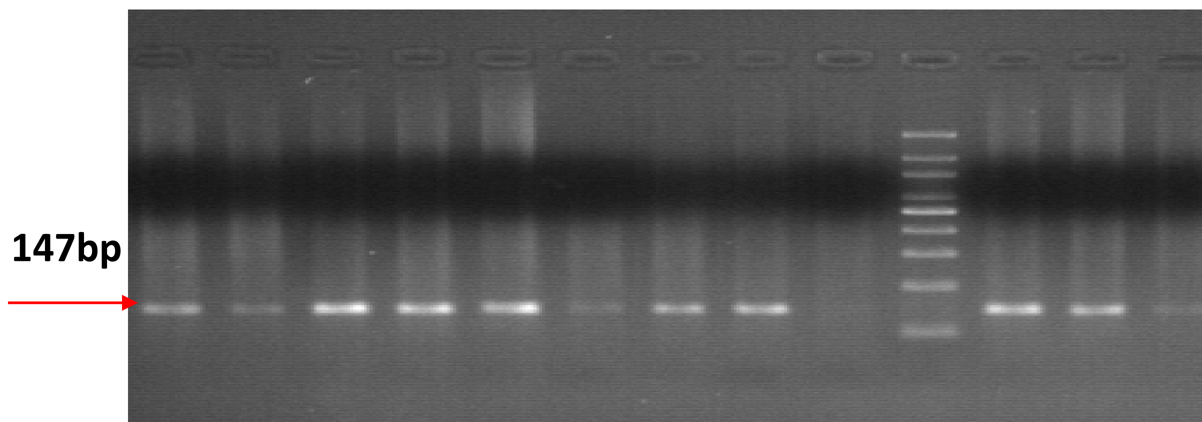

Fig. 30: Check gel for FV Leiden

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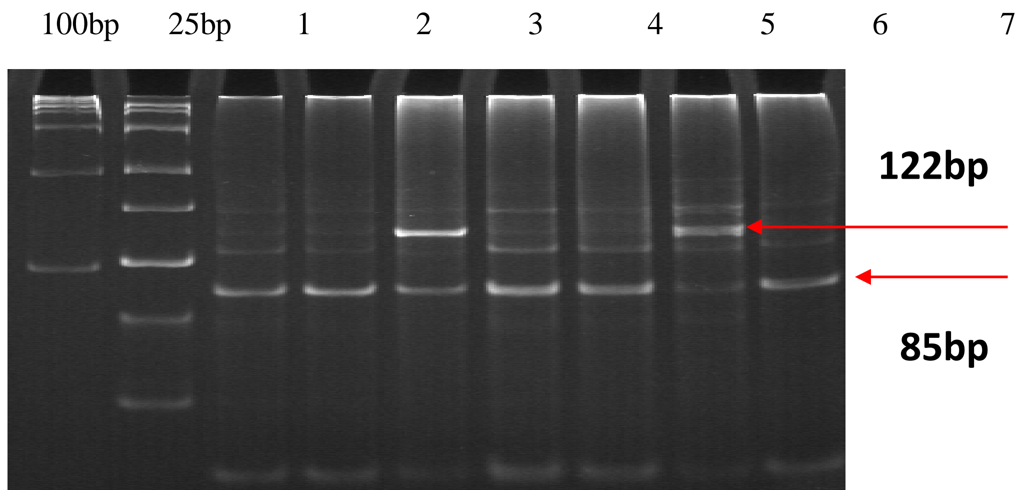

Fig. 31: Factor five Leiden mutation (lane 3, 6 heterozygous)

ii) **Methylene tetrahydrofolate reductase (MTHFR):** (Hanson et al.2001)[12]

Primer sequence

5'-CTTCTACCTGAAGAGCAAGTC-3' Fw

5'-CATGTCCACAGCATGGAG-3'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
- 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

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

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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% agrose gel

1 2 3 4 5 6 7 8 9 100bp

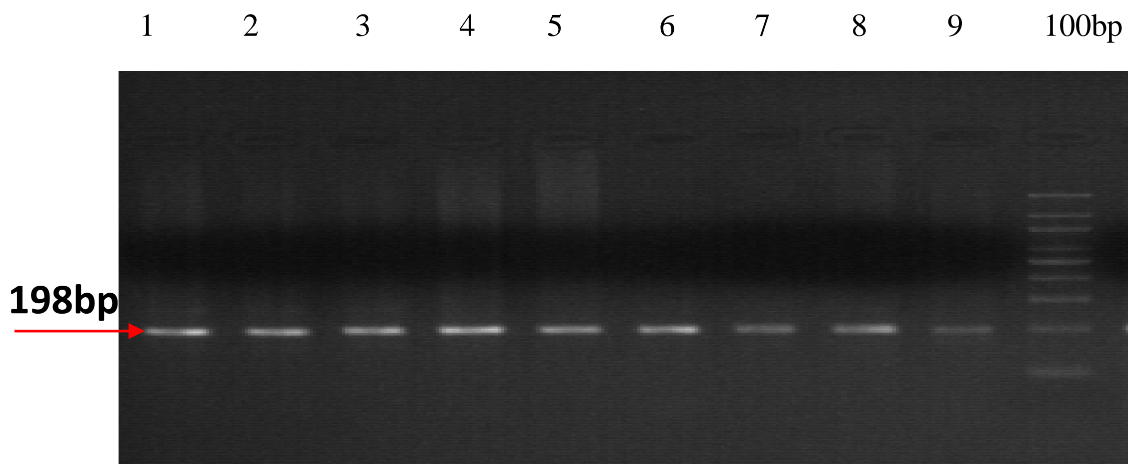

Fig.32: MTHFR check gel

25bp 1 2 3 4 5 6 7 8 9

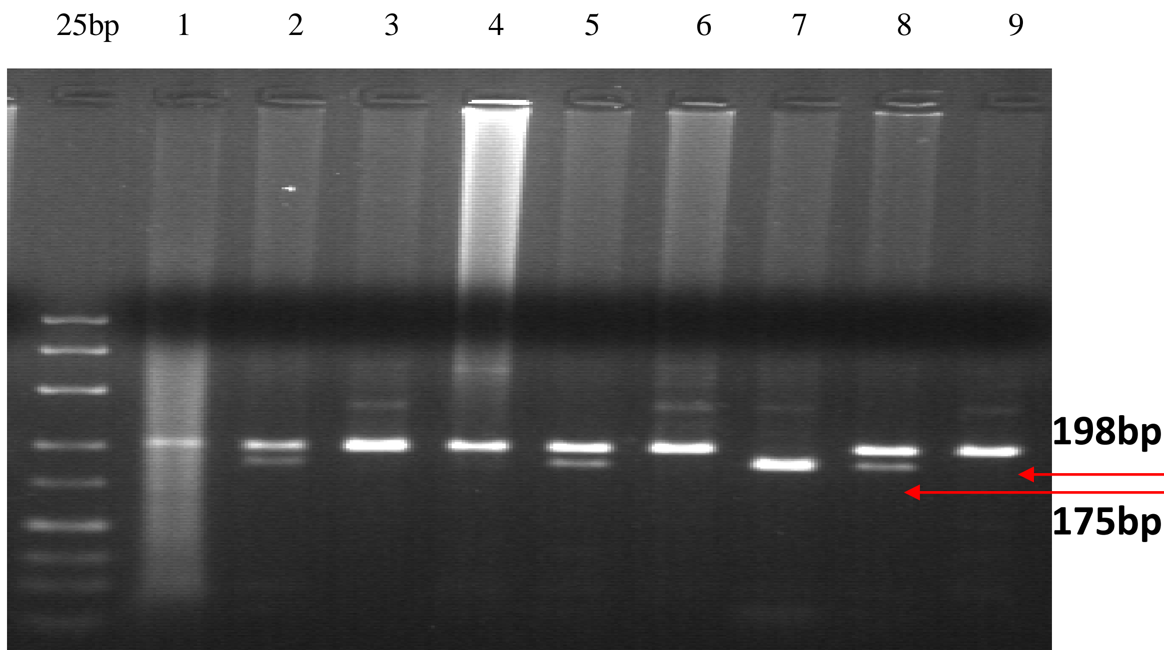

Fig. 33: Restriction digestion for MTHFR (lane 2, 5, 8 are heterozygous and lane 7 is homozygous)

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(iii)Prothrombin mutation: (Poort et al.1996)[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
- 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

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% agrose gel

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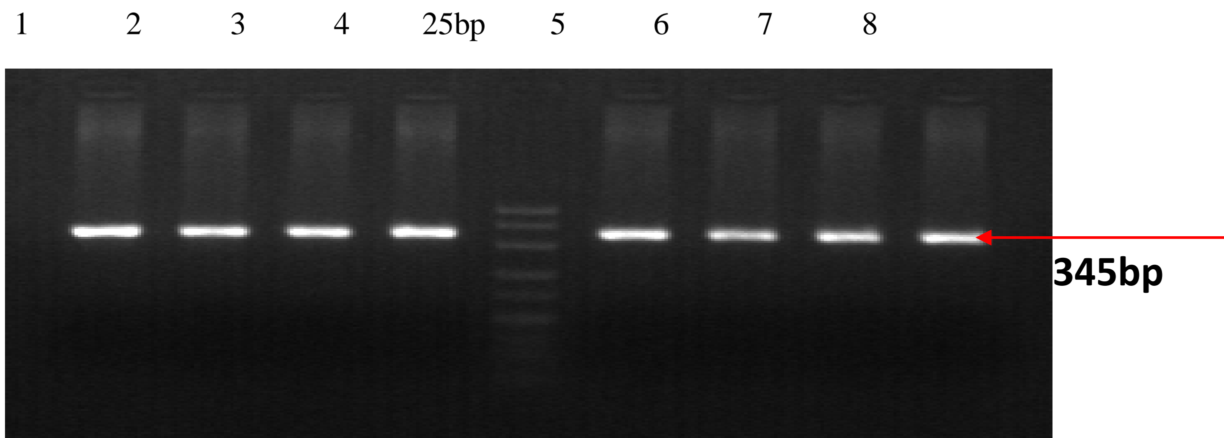

Fig. 34: Check gel for Prothrombin mutation

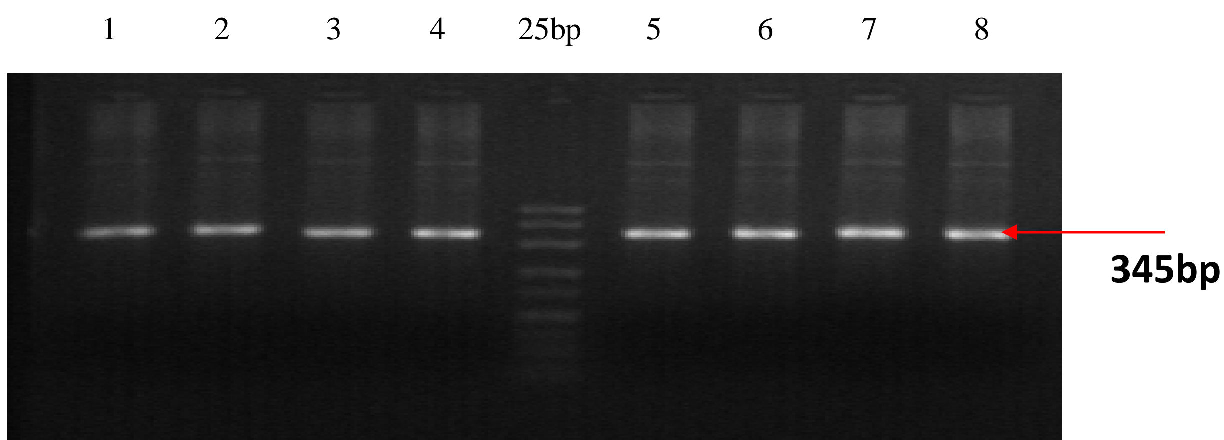

Fig. 35: Restriction digestion for prothrombin mutation (Not Digested)

CONCLUSION:

Molecular diagnosis of disease based on PCR technology should be established in contemporary, secondary & tertiary care hospitals, advanced pathological centres, and specialized clinics in all major cities catering more than 15 lac of population irrespective of their cosmopolitan and metropolitan status. PPP (Public Private Partnership) model should be followed and accepted to provide better health facility and quick diagnosis for advanced treatment for the general population. Govt should make stringent laws and MCI; ICMR; DHR should incorporate them to be followed in patient care. In modern day's world being a global village and increase in intercontinental, inter-state, inter-region, inter-race marriage which is very important for social amalgamation but also needs to be medically supervised for healthy progeny and generations to come. PCR based diagnosis is rapid, accurate and gold standard in detection of fatal disease. Advanced PCR Technology is most useful in early detection of inherited disorders and prenatal diagnosis of mutations so as to go for genetic counselling and plan a disease free healthy family life. These Diagnoses made through PCR helps in prevention of diseases through counselling; proper Disease management and thus increase life expectancy.

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Asia type and hydrops fetailis by polymerase chain reaction. Blood .1991;78: 853-854.

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