

## HEMATOGENETICS

### **Prevalence and Molecular Characterization of Red Cell Pyruvate Kinase (PK) Deficiency.**

*Years of Commencement* : 2000

*Years of Completion* : 2005

Twenty-six cases of neonatal jaundice and sixty four cases of non-spherocytic hemolytic anemia were investigated for pyruvate kinase deficiency by assaying the enzyme activity spectrophotometrically. Four cases showed a deficiency of pyruvate kinase activity (0.91 to 6.12 IU/gm Hb). With a compensatory rise by 2 to 3 fold levels and some reductions in ATP levels. The remaining cases showed normal PK activity (Range 9.98 to 15.5 IU/gm Hb).

Out of four cases two had a history of neonatal jaundice and also received phototherapy and required exchanged blood transfusion. Other causes of intracorpuscular hemolysis like presence of unstable hemoglobin, thalassemia, hereditary spherocytosis and G6PD deficiency were ruled out. The presence of autoantibodies and cold antibodies were also excluded.

Hematological and biochemical analysis in PK deficiency

	CASE 1	CASE 2	CASE 3	CASE 4
AGE/SEX	23/F	23/F	5days/F	11 months
HB	6.6	5.1	18.0	5.5
RBC	1.83	1.38	4.90	2.01
HCT	22.9	16.6	54.2	16.4
MCV	125.1	120.3	110.6	81.6
MCH	36.1	37.0	36.7	27.4
MCHC	28.8	30.7	33.2	33.5
RDW	20.4	24	17.9	14.6
RETIC	25%	17.3%		12.0%
BILI TOTAL	3.6	2.0		
INDIRECT	2.7	1.2		
G6PD	NORMAL	NORMAL	NORMAL	NORMAL
P.K.	0.91	3.26	4.61	6.12
2,3 DPG	16.80	24.92		20.4
ATP	2.14	4.0		3.5

## NORMAL RANGES

<u>PK (NEONATES)</u>	7.2 — 12.5 IU/gm Hb
PK (ADULT)	11.5 — 15.5 IU/gm Hb
2,3 DPG	8.16 — 14.7 $\mu$ mol/gm Hb
ATP	3.22 — 5.2 $\mu$ mol/gm Hb

All four cases shows absence of 1456 C -> T and 1529 C-> T mutation by DNA sequencing of Exon 11 which was found in our other cases previously reported. However other exon will be now sequenced for further characterization.

### **Glucose-6-phosphate Dehydrogenase mutations and haplotypes in various ethnic groups of India.**

*Year of Commencement : 2004.*

*Year of Completion : 2007.*

The Indian population comprises of numerous caste and tribal groups, each with characteristic physical, cultural, and genetic backgrounds. Since India is endemic for malaria, G6PD deficiency is found in most of the populations. Considering the diversity of the Indian population, it is highly probable that many variants of G6PD deficiency may exist in the Indian sub-continent. However, there is little information about the mutations causing G6PD deficiency and their distribution among the diverse Indian populations. The aims and objectives of the present study are:

- ❑ To characterize the mutations underlying the G6PD deficiency.
- ❑ To see the distribution of these mutations in different population groups of India.
- ❑ Studying different polymorphic sites in the G6PD gene (Haplotype) to define the genetic framework as well as the origin of different mutations.

A total of 72 G6PD deficient individuals from the tribal groups of Maharashtra and Tamilnadu were screened for mutational analysis. Initially all the samples were screened for G6PD Orissa (131C→G) mutation in exons 3 and 4 by PCR-RFLP analysis. When this mutation was found to be absent then the samples were tested for G6PD Mediterranean (563C→T) and G6PD Kerala Kalyan (949 G→A) mutations in exons 6, 7 and 9 respectively. Table 1 shows the number of G6PD deficient samples from Maharashtra and Tamilnadu and the distribution of G6PD mutations in different tribal groups.

In Maharashtra, out of 32 G6PD deficient samples, 17 were from Madia tribe of Gadchiroli district and 15 were from Kolam tribe of Yavatmal district. G6PD Orissa was found to be very common in both the groups with an overall prevalence of 59.3% followed by G6PD Kerala Kalyan (9.3%). None of the individuals showed the presence of G6PD Mediterranean mutation. Hence, ten samples (31.4%) did not show the presence of any of the three mutations. Further characterization of these samples is in progress.

In Tamil Nadu, out of 40 G6PD deficient samples 13 were from Irula tribe, 15 were from Kurumba tribe, 11 from Molukurumba tribe and 1 from Paniya tribe of Nilgiri district. During screening for G6PD Orissa mutation (131 C→G) by Hae III restriction enzyme digestion it was found that only one individual (2.5%) had this mutation whereas in 25 (62.5%) individuals the expected 107 bp band was absent (Fig 1.)

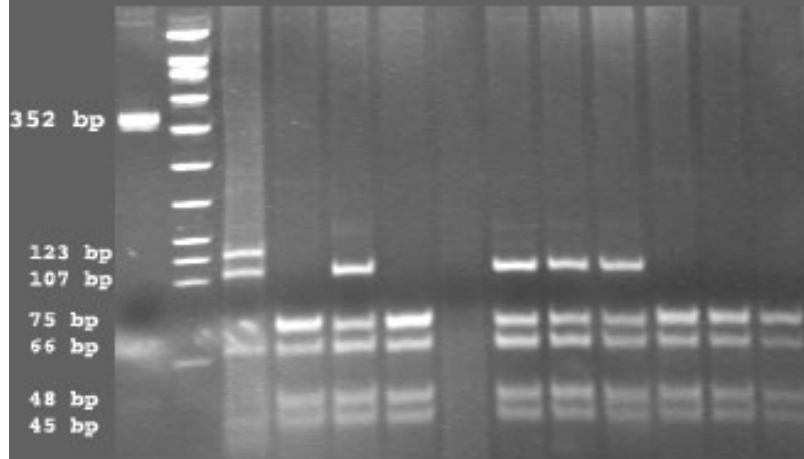
DNA sequencing of these samples revealed a T→C substitution at position 208 in exon 4, resulting in the substitution of histidine for tyrosine at amino acid 70 which is responsible for G6PD Namoru mutation (Fig 2.) Creation of an Nla III restriction site (207 bp and 113 bp) has also been observed in these samples, (Fig.3) which further confirms the presence of the G6PD Namoru mutation. G6PD Namoru is a very rare mutation and has been reported in three G6PD deficient individuals from Vanuatu Archipelago (South Western Pacific Region). None of the remaining 14 samples (35.0%) showed the presence of G6PD Mediterranean mutation. Further characterization of these samples is in progress.

**Table 1: Distribution of G6PD mutations in the different tribal groups**

STATE	DISTRICT	TRIBES	TOTAL	G6PD Mediterranean (563C→G)	G6PD Orissa (131C→G)	G6PD Kerala kalyan (949G→A)	G6PD Namoru (208T→C)
Maharashtra	Gadchiroli	Madia	17	0	12(70.5%)	2(11.7%)	--
	Yavatmal	Kolam	15	0	7(46.6%)	1(6%)	--
		TOTAL	32	0	19(59.3%)	3(9.3%)	--
Tamilnadu	Nilgiri	Irula	13	0	0	NT	7 (53.8%)
	Nilgiri	Kurumba	15	0	0	NT	9 (60%)
	Nilgiri	Molukurum	11	0	1	NT	9 (81.8%)
	Nilgiri	Paniya	1	0	0	NT	--
		TOTAL	40	0	1 (2.5%)	--	25 (62.5%)

NT: Not tested

Fig 1: Hae III restriction enzyme digestion in 10% polyacrylamide gel



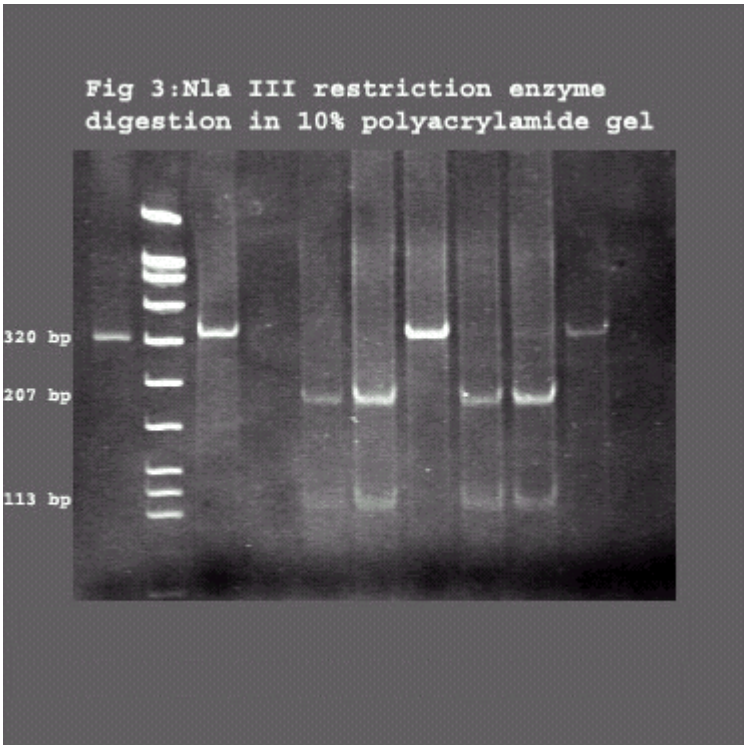
Lane 1: Amplified undigested DNA

Lane 2: Molecular Weight Marker VIII

Lane 3: Digested DNA showing G6PD Orissa mutation

Lane 5, 8, 9, 10 : Digested DNA not showing G6PD Orissa mutation

Lane 4, 6, 11, 12, 13: Digested DNA showing the absence of 107 bp band.

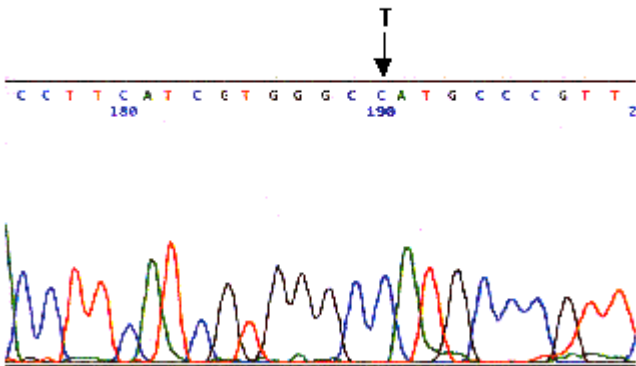


Lane 1 : Amplified undigested DNA

Lane 2 : Molecular Weight Marker VIII

Lane 3, 7, 10 : Digested DNA not showing G6PD Namoru Mutation

Lane 5, 6, 8, 9 : Digested DNA showing G6PD Namoru Mutation



**Figure 2 :DNA sequencing of exon 4 showing G6PD Namoru mutation at 208 T→C (70 Tyr→His)**

**Isolation of fetal cells and cell free fetal DNA from maternal blood for Non-Invasive Prenatal Diagnosis of Hemoglobinopathies**

*Year of commencement: 1999*

*Year of completion: Ongoing*

The project of establishing the non-invasive prenatal diagnosis of hemoglobinopathies by isolating fetal cells from maternal blood and circulatory cell free DNA from maternal plasma was continued.

**Fetal nucleated RBCs from maternal blood:**

The isolation based on Fluorescence Activated Cell Sorting was carried out using 3 monoclonal antibodies CD45, Glycophorin A and Human fetal hemoglobin.

We have continued with the modified protocol as described last year. The enrichment of the fetal cells was done by using a discontinuous Percoll density gradient. On enrichment the cells were permeabilized using glutaraldehyde and then incubated with CD45 per CP. After a CD45 negative sort, the sorted cells were then incubated with Glycophorin A tagged with Phycoerythrin (PE) and Hb F tagged with FITC to isolate the fetal NRBCs.

The results are shown below:

Sr no	Gestation age (Weeks)	Presumptive no. of nRBC per 10000 CD45 negative cells	Percentage of fetal NRBC
1	10	65	0.01
2	10	67	0.01
3	19	47	0.008
4	9	67	0.03
5	19	74	0.03
6	12	32	0.016
7	12	90	0.036
8	10	219	0.022
9	11	590	0.088
10	11	273	0.081
11	12	106	0.047
12	12	25	0.035

These isolated fetal cells were then used for further genetic analysis.

The DNA was isolated from the fetal cells by using the QIAMP Blood Kit (Qiagen) and used for genetic analysis. The mutations causing the hemoglobinopathies in the parents was first characterized using a stepwise approach beginning with the screening for the six common Indian mutations along with Hb S and Hb E by Covalent Reverse Dot Blot Hybridization. The rare  $\beta$ -thalassemia mutations were characterized by Amplified Refractory Mutation System.

**Standardization of a nested PCR approach for identification of mutations in the fetal DNA isolated from the maternal blood:**

The mutations in the fetal DNA were characterized in four cases. The approach used was based on where the mutation lies in the  $\beta$ -globin gene in the parents.

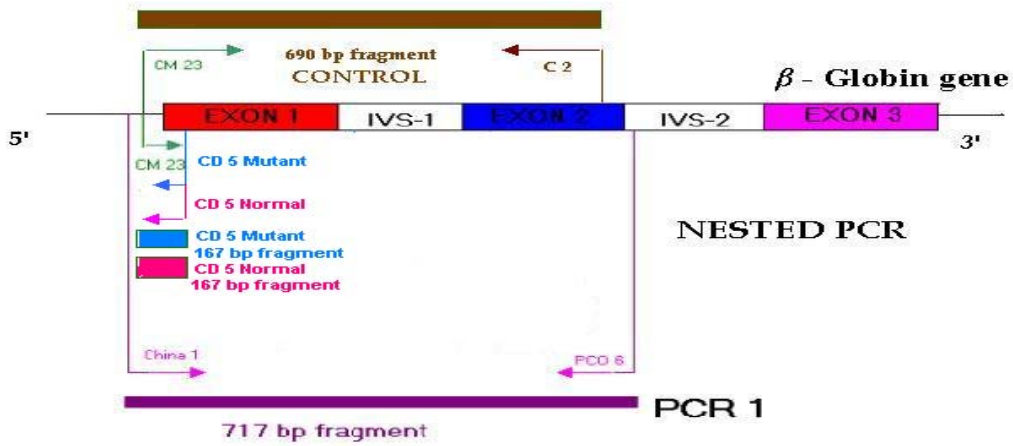
In three of the cases the first PCR was carried out by amplifying a 717 bp fragment, 10 microlitre of the fetal DNA was used as the template. A nested PCR was then performed using the above PCR product. We have standardized the quantity of the amplified DNA required for the nested PCR. A 1:100 dilution of the first PCR product gave best results. The nested PCR used was based on ARMS. Here depending on the mutation obtained in the parents, we screened the fetal DNA for the same mutation/s.

In the fourth case, since the paternal mutation was the 619 bp deletion, in the first PCR we amplified a 1.2 kb fragment across the deletion. In the nested PCR we amplified a smaller 860 bp fragment. If the deletion is present then we get a 241 bp fragment .

The results were as follows:

Sr. No.	Mother's Mutation	Father's Mutation	Mutation detected in the CVS	Mutation detected in the Isolated Fetal Cells
1	Codon5(-CT) heterozygous	Codon5(-CT) heterozygous	Normal	Codon 5 (-CT) absent. Therefore normal.
2	IVS 1 nt 5 (G→C) heterozygous	Codons 41/42 (-CTTT) heterozygous	IVS 1 nt 5 (G→C) heterozygous	IVS 1 nt 5 (G→ C) present Codons 41/42 (-CTTT) absent.
3	IVS 1 nt 5 (G→C) heterozygous	IVS 1 nt 5 (G→C) heterozygous	IVS 1 nt 5 (G→C) heterozygous	IVS 1 nt 5(G→C) heterozygous.
4	Normal	619 bp deletion heterozygous	619 bp deletion heterozygous	619 bp deletion present.

CASE I



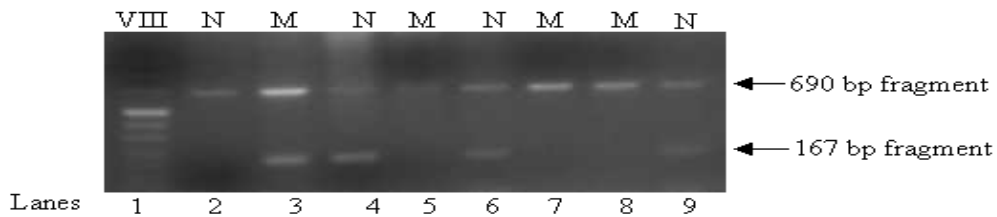
PCR I – 717 base pair (Promoter to mid of IVS 2)

PCR II the Nested PCR – Amplified Refractory Mutation System (ARMS)

Control band = 690 bp fragment

Codon (-CT) = 167 bp fragment

Result:



Key: N = Normal M= Mutant

ARMS PCR to screen for Codon 5 (-CT) heterozygous

Lane1 Marker VIII (Roche)

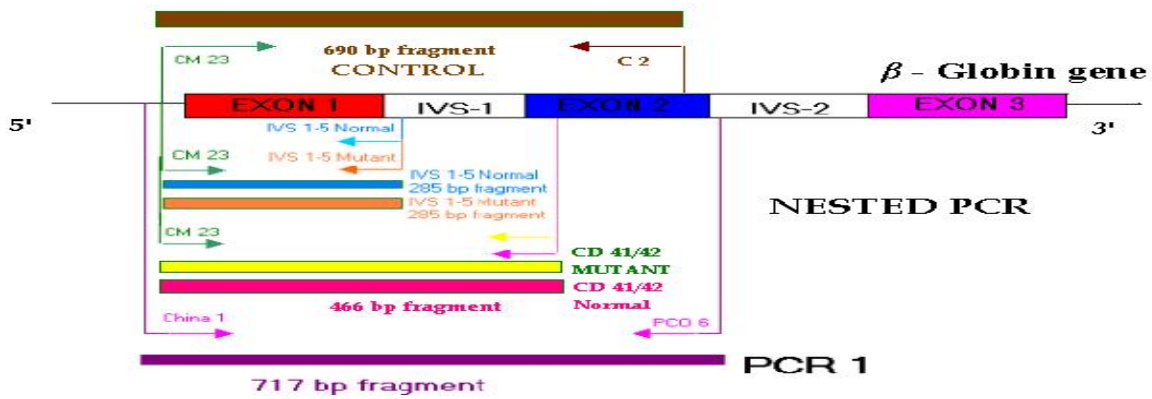
Lanes 2 & 3 Mother Codon 5 (-CT) heterozygous

Lanes 4& 5 DNA from Fetal cells Normal

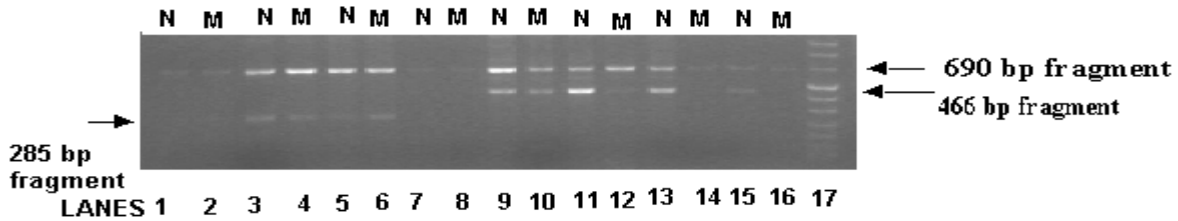
Lanes 6& 7 CVS Normal

Lanes 8& 9 Negative control Codon 5 (-CT) absent

CASE II



PCR I – 717 base pair (Promoter to mid of IVS 2)  
 PCR II the Nested PCR – Amplified Refractory Mutation System (ARMS)  
 Control band = 690 bp fragment  
 IVS1-5 (G→C) = 285 bp fragment  
 Codon 41/42 (-CTTT) = 466 bp fragment



Result:

ARMS PCR to screen for IVS 1 nt 5 (G→C) mutation

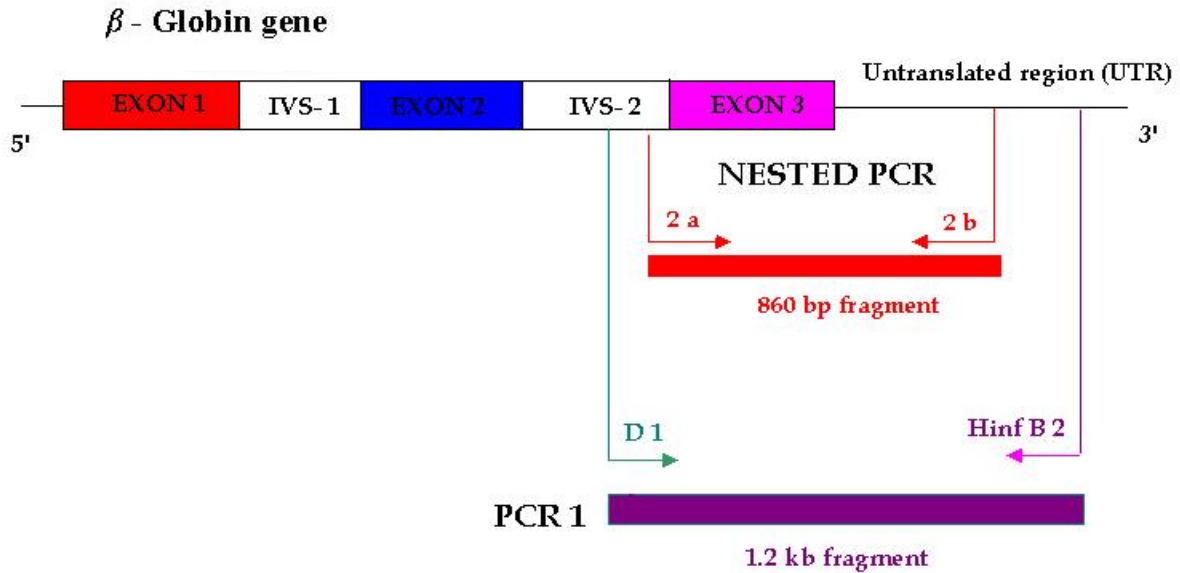
1 & 2	Mother	IVS 1 nt 5 (G→C) heterozygous
3 & 4	DNA from Fetal cells	IVS 1 nt 5 (G→C) heterozygous
5 & 6	CVS	IVS 1 nt 5 (G→C) heterozygous
7 & 8	Father	CD 41/42 (-CTTT) heterozygous

ARMS PCR to screen for CD 41/42 (-CTT) mutation

9 & 10	Father	CD 41/42 (-CTTT) heterozygous
11 & 12	DNA from Fetal cells	CD 41/42 (-CTTT) absent
13 & 14	CVS	CD 41/42 (-CTTT) absent
15 & 16	Mother	IVS 1 nt 5 (G→C) heterozygous

Lane 17 : Marker VIII

CASE IV

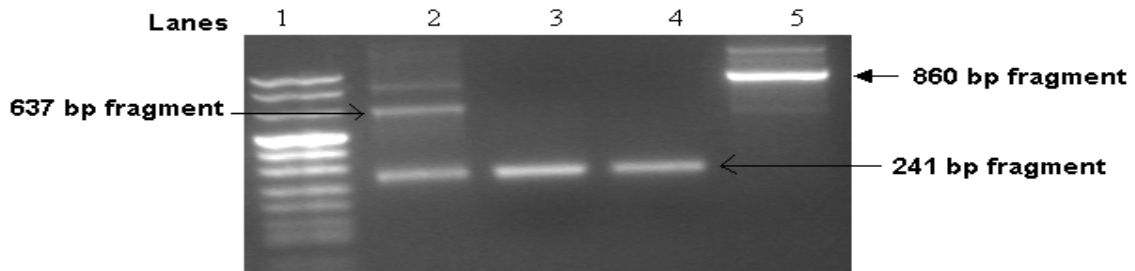


PCR I – 1.2 kb

PCR II the Nested PCR – Amplified Refractory Mutation System (ARMS)

Control band = 860 bp fragment

Result:



ARMS Nested PCR to screen for 619 bp deletion

Lane 1	Marker VIII	
Lane 2	Father	619 bp deletion
Lane 3	DNA from Fetal cells	619 bp deletion
Lane 4	CVS	619 bp deletion
Lane 5	Mother	Normal

## Circulatory Cell Free Fetal DNA

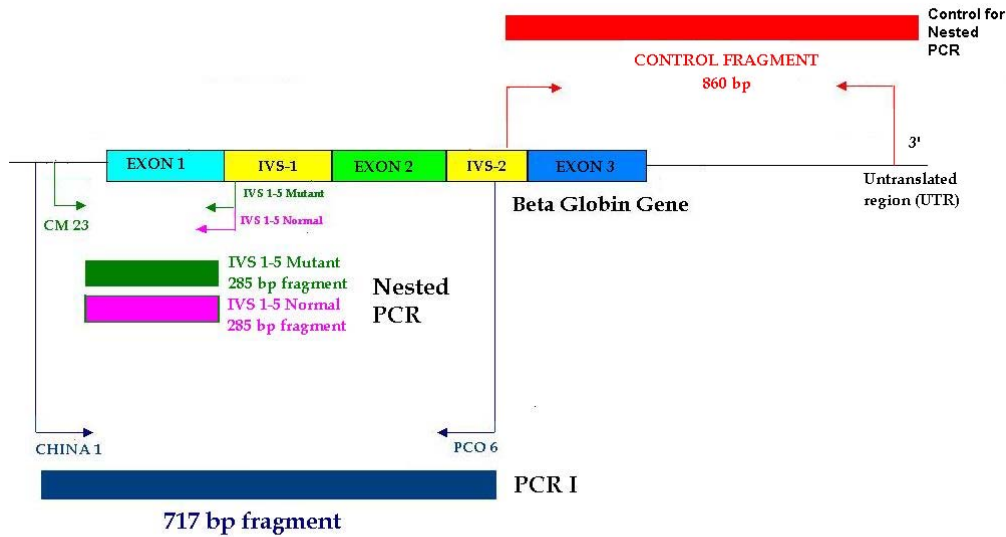
A similar approach was carried out in the non-invasive prenatal diagnosis using maternal plasma to isolate the circulatory cell free DNA. The samples were chosen wherein the maternal and paternal mutations were different. The plasma was obtained by centrifuging the peripheral venous blood at 3000 rpm for 10 minutes. This plasma was then recentrifuged at 3000 rpm for 10 minutes. The cell free DNA was then extracted from the plasma using QIAMP Blood kit (Qiagen). A similar nested PCR approach, as that used for the fetal cells, was used to characterize the mutations in 3 cases. The only difference being that here we screened the cell free fetal DNA only for the presence or absence of the paternal mutation.

The results were as follows:

Sr. N	Mother's Mutation	Father's Mutation	Mutation detected in the CVS	Mutation detected in the Plasma DNA
1	619 bp deletion heterozygous	IVS 1 nt 5 (G→C) heterozygous	IVS 1 nt 5 (G→C) heterozygous	Presence of IVS 1 nt 5 (G→C) shown
2	IVS 1 nt 5 (G→C) heterozygous	Codon 16 (-C) heterozygous	IVS 1 nt 5 (G→C) heterozygous Codon 16 (-C)	Presence of Codon 16 (-C) shown
3	IVS 1 nt 5 (G→C) heterozygous	Codons 8/9 (+C) heterozygous	Codons 8/9 (+C) heterozygous	Presence of Codons 8/9 (+C) shown

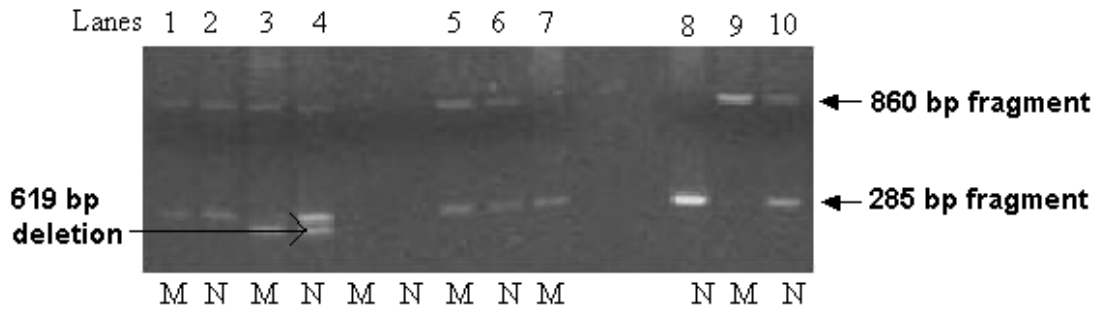
We have been able to detect the presence of the paternal mutation in all the three cases, however prenatal diagnosis can only be given in cases when the paternal mutation is absent.

CASE I



PCR I – 717 base pair (Promoter to mid of IVS 2)  
 PCR II the Nested PCR – Amplified Refractory Mutation System (ARMS)  
 Control band = 690 bp fragment  
 IVS1-5 (G→C) = 285 bp fragment

Result:



ARMS PCR to screen for IVS 1 nt 5 (G→C) mutation in the cell free DNA from maternal plasma

1 & 2	Father	IVS 1 nt 5 (G→C) heterozygous
3 & 4	Mother	619 bp deletion
5 & 6	CVS	IVS 1 nt 5 (G→C) heterozygous
7 & 8	Fetal plasma DNA	IVS 1 nt 5 (G→C) heterozygous
9 & 10	Negative control	IVS 1 nt 5 (G → C) Absent

## **WHO project on “Community Control programme for Hemoglobinopathies.”**

Year of Commencement- 2002

*Year of Commencement- 2004*

### **Aims & Objectives**

The main aim of this pilot study was to generate awareness on thalassemia and sickle cell disease in 3 states of India, viz. Chattisgarh, Bihar and Madhya Pradesh by focusing on pregnant women in antenatal clinics and to establish centres for education, screening and genetic counselling at Raipur, Patna and Jabalpur. I.I.H. Mumbai was the co-ordinating centre.

The specific objectives were as under

1. To educate women attending the antenatal clinics in maternity hospitals in the three states to register early for the first antenatal check-up.
2. To screen the women and identify carriers of  $\alpha$ -thalassemia and other abnormal hemoglobins.
3. To call the couple and give them information on this disorder.
4. To test the husbands whenever required to identify couples at risk of having a child with major disease.
5. To offer genetic counselling and prenatal diagnosis whenever required.
6. Create awareness in the general public about these disorders and the need for screening to identify carriers.
7. Evaluation of the feasibility of this approach for preventing the birth of an affected child.

These groups will also be screened for iron deficiency by estimation of protoporphyrin levels (ZPP).

This project has been completed and the work done is summarized below:

### **Preparation of Educational Material**

Educational booklets in Hindi and English were prepared on Thalassemia and Sickle cell disease by I.I.H., Mumbai and sent to the 3 centres at Raipur, Patna and Jabalpur. The proformas

consent forms and an information sheet on Thalassemia were also prepared and printed at Mumbai and sent to the 3 centers.

A laboratory manual giving details of all the procedures was prepared by I.I.H. Mumbai for the 3 centers. This also included instructions for filling up of the proformas.

A DVD on "Prevention of Thalassemia" for Awareness Generation in the General Population was also prepared under the guidance of I.I.H. , Mumbai by the students of Mass Communication, Sophia College, Mumbai.

### **Workshop for Training of Staff:**

The laboratory technicians of the 3 centres were given training in the methodology to be used from 14<sup>th</sup> to 18<sup>th</sup> June 2004. They were given hands on experience on all the procedures to be used with stress on quality control. This was followed by discussion on problem solving as well as some theoretical background. They were also given detailed instructions for filling up the proformas correctly.

### **Awareness and Screening Programmes:**

The techniques were then standardized at the 3 centres and after contacting different antenatal clinics; screening of pregnant women was initiated.

A total of 1108 women have been screened at the 3 centres, Hematological indices on the cell counter, NESTROFT, Hemoglobin electrophoresis on cellulose acetate and quantitation of Hb A<sub>2</sub> by elution and estimation of ZPP for screening for iron deficiency were done locally and an aliquot of the samples were sent to I.I.H, Mumbai for analysis by automated HPLC on the Variant Hb Testing System (Biorad) for confirmation of diagnosis.

The different hemoglobinopathies detected at the 3 centres are shown in Table 1.

The prevalence of  $\alpha$ -thalassemia trait was 3.0% at Raipur, 2.5% at Jabalpur and 1.9% at Patna. The prevalence of sickle cell trait was 5.8% at Raipur and 6.5% at Jabalpur. HbS was not seen in Patna. 3 cases of Sickle Cell Disease were also found in Raipur. Occasional cases of HbD, HbE and raised HbF ( $\alpha\alpha$  Thalassemia/HPFH) were also detected. Two other abnormal hemoglobins were detected which have yet to be characterized.

Table 2 shows the number of pregnant women having iron deficiency based on screening by estimation of zinc protoporphyrin (ZPP>80  $\mu$ mols/mol Heme). The prevalence of iron

deficiency was 40.6% at Raipur, 31.1% at Jabalpur and 49.8% at Patna. All the samples collected could not be screened for iron deficiency as the hematofluorimeters were installed a few months after commencement of screening.

At Raipur and Jabalpur all the  $\alpha$ -thalassemia traits had low MCV (<75 fl) and low MCH (<25pg). However at Patna 2 of the 4  $\alpha$ -thalassemia traits had an MCH of >25.

There were no discrepancies between the results of Hb electrophoresis and HPLC (Variant) for identification of the Sick cell heterozygotes. However at Raipur and Patna, most of the  $\alpha$ -thalassemia heterozygotes were not correctly picked up by Hb electrophoresis (Table 3)

### **Genetic Counselling and Screening of Husbands of Heterozygous Women.**

Majority of the pregnant ladies were in the 2<sup>nd</sup> and 3<sup>rd</sup> trimester when they came to the antenatal clinics at all the centres. Counselling was given but screening of husbands was not possible. At Raipur, letters were also sent to all the  $\alpha$ -Thalassemia trait and sickle cell trait ladies but only one husband came for testing and he was normal. At Jabalpur, 16 husbands were tested and one of them had Sickle cell trait. This couple already had a 5-year-old child with sickle cell disease but were counseled for future pregnancies. All the other husbands were normal.

Due to shortage of time further follow-up and home visits were not possible.

### **Workshop on Hemoglobinopathies at Raipur**

A workshop was organized by Institute of Immunohaematology at the collaborative centre at Raipur from 29<sup>th</sup> to 31<sup>st</sup> October 2004 for 30 medical officers and 45 technicians from all the districts of Chattisgarh as well as the Principal Investigators, Medical Officers and Technicians from our other two collaborative centres Jabalpur and Patna. Lectures by eminent scientists from different parts of the country on laboratory diagnosis, quality control, prevalence, clinical presentation and management of sickle cell disease in particular were arranged. The Joint Director, Health Services, Maharashtra and Additional Director, Health Services Gujrat also participated and presented the work done in their states on Sickle cell disease and the measures taken for control of the disease.

The laboratory technicians were given hands on training on screening procedures like NESTROFT, Solubility test and Hb electrophoresis. The medical officers were shown interesting cases and they discussed the clinical problems and management strategies with the

faculty. All the participants were given the proceedings of the lectures and laboratory protocols to be followed.

**Table 1 : Hemoglobinopathies detected at Raipur, Jabalpur and Patna**

Centre	No. of women screened	b-thal trait	Sickle Cell Trait	Sickle Cell Disease	Hb D trait	Hb E trait	db thal / HPFH Trait	Uncharacterized Hb
Raipur	500	15 (3.0%)	29 (5.8%)	3 (0.6%)	1	1	1	1 (Retn time 17.2)
Jabalpur	397	10 (2.5%)	26 (6.5%)	0	1	1	1	1 (Retn time 2.68)
Patna	211	4(1.9%)	0	0	0	1	0	--

**Table 2 : Iron Deficiency in Pregnant Women at Raipur, Jabalpur and Patna.**

Centre	No. of women screened	No. of women having iron iron deficiency (ZPP>80 mmol/mol Heme)
Raipur	293	119 (40.6%)
Jabalpur	225	70 (31.1%)
Patna	211	105(49.8%)

**Table 3 : Discrepancy in Diagnosis of  $\alpha$ -Thalassemia Trait(HbA2 Estimations) by Hb electrophoresis and HPLC (Biorad Variant)**

Centre	No. of Samples	$\alpha$ -thal trait by Hb Electrophoresis and Normal by HPLC	Normal by Hb Electrophoresis and $\alpha$ -thal Trait by HPLC
Raipur	500	4	9
Jabalpur	397	0	1
Patna	211	52	3

**Table 4 : Women with hemoglobinopathies identified during different trimesters of pregnancy**

Centre	Hemoglobinopathies detected	I <sup>st</sup> Trimester	II <sup>nd</sup> Trimester	III <sup>rd</sup> Trimester	Total
Raipur	$\alpha$ -thal trait	3	7	5	15
	HbS trait	2	11	15	28
	HbS	0	1	2	3
	homozygous	0	1	0	1
	HbD trait		0	1	1
	Unknown				
Patna	$\alpha$ -thal trait	2	1	1	4
	Hb E trait	1	0	0	1
Jabalpur	$\alpha$ -thal trait	0	1	2	3
	HbS trait	0	7	5	12
Total		8	29	31	68

**Molecular characterization of  $\beta$ -thalassemia and hereditary persistence of Hemoglobin (HPFH)**

*Year of commencement* : 2001

*Year of completion* : On going

The study was continued to look for the molecular lesions leading to raised HbF phenotypes in adult life. The gap PCR based approach was used to screen these individuals. 20 cases from diverse ethnic groups were studied. Nine individuals from this group were hetetozygotes for the Asian Indian inversion deletion  $G\gamma$  ( $A\gamma\delta\beta$ ) thalassemia. 4 cases remained uncharacterized.

**Hematological and molecular analysis of these cases is shown in table 1 and 2.**

**Table 1 : Molecular and Hematological analysis of high Hb F determinants**

	HPFH –3	Deletional inversion
	Mean (Range)	Gr ( $Ar\delta\beta$ ) <sup>0</sup> thal
		Mean (Range)
Subjects	9	7
RBC ( $10^6/\mu\text{l}$ )	4.52 (3.2 – 5.8 )	4.83 (4.2 – 5.3 )
Hb ( g / dl )	13.5 (10.2 –15.6)	12.6 (9.5 – 14.3)
MCV (fl )	79.0 (72 – 94.2 )	67.4 (61.3 – 73 )
MCH ( pg )	27.3 (23.1 – 29.1)	22.8 (20.8 – 24.9)
Hb A <sub>2</sub> (%)	2.5 (2. 1 – 3.5 )	2.3 ( 2. 1 - 3.8 )
Hb F ( % )	29.6 ( 17 – 35. 2)	18.2 ( 14.1 – 22. 6)

**Table 2 : Hematological analysis of 4 uncharacterized samples**

Age Sex	Caste	Origin	RBC (10 <sup>6</sup> /μl)	Hb (g / dl )	MCV (fl)	MCH ( pg )	Hb A <sub>2</sub> (%)	Hb F (%)
22/F	Kunbi	Ratnagiri	4.29	11.3	78.1	26.3	2.1	18.3
14/F	Jain	Gujrat	4.97	9.6	59.4	19.3	3.4	11.3
41/M	Budha	Kolhapur	5.15	12.4	70.7	24.1	2.5	30.3
27/F	SC	Sindhudurg	4.22	9.1	64.9	21.6	3.2	11.2

## **The Effect Of Hydroxyurea On The Fetal Hemoglobin Response And Phenotypic Expression Of Sickle Cell Disease**

*Year of Commencement* : 2004

*Year of Completion* : Ongoing

This project was sanctioned under the human genome programme and was initiated last year.

Sickle cell disease has an extremely varied clinical presentation in India. Being linked to the Arab-Indian haplotype, the disease is relatively mild in the tribal populations but a large number of cases have severe manifestations particularly in the scheduled caste population. Recurrent painful crises often lead to end organ damage, a poor prognosis and mortality.

Hydroxyurea (HU) is one drug that has been shown to benefit severe cases by its ability to reactivate HbF synthesis. This has been used in other population groups successfully. However, the response to HU is variable depending on the genetic background on which the sickle mutation has arisen. No systematic study has been conducted to determine this response in Indian sicklers.

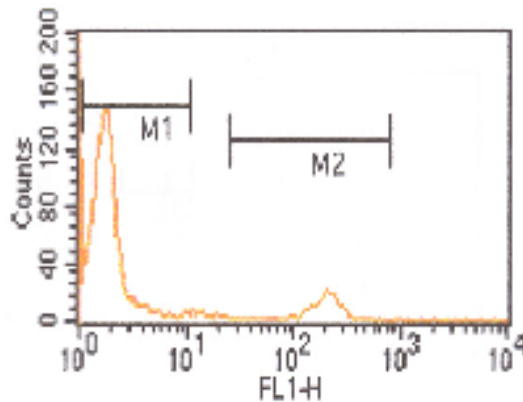
This study was thus started with the following objectives.

- ✓ To evaluate the effect of HU on HbF response in severe cases.
- ✓ To determine the contribution of polymorphisms in the  $\beta$  globin gene cluster in relation to the HbF response.
- ✓ To evaluate the clinical benefits of HU therapy in these cases.
- ✓ To see the effect of different concentrations of HU on F-cell response by cell culture studies.

**Standardization of estimation of F-cells by Flow cytometry**

This method was standardized using a monoclonal anti HbF antibody tagged with FITC (figure1) and was compared with the traditional Kleihauer-Betke’s Slide Method of F-cell estimation and HbF estimation using the Variant Hb Testing System in 100 samples comprising of the following groups– 25 Normal individuals, 25 Sickle heterozygotes, 25 Sickle homozygotes and 25 Sickle β-thalassemia cases.

**Figure 1: Estimation of F-cells by Flow cytometry**



**Table of Mean F-cells and HbF levels in different groups**

Sr no	Technique	F cells				Hb F	
		KB		Flow cytometry		HPLC (Variant)	
		Mean	± SD	Mean	± SD	Mean	± SD
1	Normal	1.24	± 0.78	4.54	± 2	0.24	± 0.3
2	S heterozygous	5.04	± 4.6	14.12	± 7.6	1.44	± 7.6

3	S homozygous	64.2	± 19.7	75.88	± 17	23.02	± 9.2
4	S Beta- thalassemia	46.3	± 15.1	56.4	± 16	19.52	± 8.5

The flow cytometric method being more accurate with objective analysis and eliminating any manual error as compared to KB was able to pick up a relatively greater number of F-cells than the visual interpretation test.

**Criteria used while selecting patients with Sickle Cell Anemia/Sickle-β thalassaemia for Hydroxyurea administration is:**

- More than 5 episodes of painful crisis per year.
- Central Nervous System affected at any time.
- Acute Chest Syndrome more than twice during lifetime.
- Avascular Necrosis of the Femur head.

Patients with any one of the above criteria were selected.

It is planned to initially include 10-15 adults and 10-15 children and follow them up periodically for a period of 2 years.

The adult patients are studied in collaboration with Hematology department of KEM Hospital and pediatric cases in collaboration with the Pediatric Department of Nagpur Medical College, Nagpur.

**Dose of Hydroxyurea**

Adult group: 500mg per day

Pediatric group: 500mg per day

The investigations done periodically include the following:

- ❑ Clinical Examination
- ❑ Liver function test

- Hematological analysis- Peripheral smear, reticulocyte count, RBC indices, WBC count
- Hemoglobin analysis by HPLC on the Variant Hb Testing System
- Quantitative estimation of F-cells by flow cytometer and Kleihauer Betke's (KB) method
- Estimation of Zinc Protoporphyrin (ZPP) using a hematofluorimeter and serum ferritin by ELISA

Molecular studies include:

- Confirmation of the sickle status by Dde I digestion and  $\beta$ -thalassemia mutation by reverse dot blot hybridization or ARMS.
- Xmn I polymorphism by restriction enzyme digestion.
- Haplotype analysis by PCR and restriction enzyme digestion.
- Sequencing of polymorphic motifs in the silencer and enhancer regions of the  $\beta$  and  $\gamma$  globin genes on the ABI Prism 310 DNA Sequencer.

6 patients from the pediatric group, 2 of whom had a history of Acute Chest Syndrome along with transfusion requirements and 4 suffered episodes of painful crises more than 5 times a year, were followed up since the last 12 months and showed an increase in HbF without any episodes of crises after the commencement of the treatment.

The number of crises have been reduced among the 3 adult patients, 2 of whom suffered episodes of painful crises more than 5 times a year and 1 had a history of Acute Chest Syndrome. They have been followed up since the last 5 months. Case no. 1 showed a fall in HbF during the 2<sup>nd</sup> month of treatment followed by an increase later and also had a moderate episode of painful crisis during the 5<sup>th</sup> month.

The results below show the comparison of the hemoglobin level, F-cell by flow cytometry, HbF by HPLC and reticulocytes count at the baseline level and after treatment with HU.

**Results:**

**Results of the baseline data and data 12 months after treatment among the pediatric group**

S. No.		PATIENT	AGE (years)	Hb Abnormality	Hb (g/dl)	RETIC (%)	F-cell (%)	Hb F (%)	Xmn I
1	Base line	N N	10	SS	6.1	9	61	15.2	+ / +
	Follow up	N N			6.5	2	75	20.0	
2	Base line	J K	8	SS	7.5	13	44	11	+ / -
	Follow up	J K			8.2	1	71	15.1	
3	Base line	R G	12	SS	9.5	3	67	16.4	
	Follow up	R G			9.7	2	82	23.3	
4	Base line	K	13	SS	7.6	10	61	16.1	+ / +
	Follow up	K			8.1	3	80	19.8	
5	Base line	M K	11	S-Beta	7.2	5	75	34.7	+ / -
	Follow up	M K			8.8	3	86	41.9	
6	Base line	N M	8	SS	7.4	18	82	32	+ / +
	Follow up	N M			9.8	8	87	30.2	

**Results of the baseline data and data 5 months after treatment among the adults**

S. No.		PATIENT	AGE (years)	Hb Abnormality	Hb (g/dl)	RETIC (%)	F-cell (%)	Hb F (%)	Xmn I
1	Base line	S K	18	S-Beta	3.2	20	73	26.7	+ / +
	Follow up	S K			9.5	4	67	25.7	
2	Base line	K F	21	SS	9.5	10	78	20.9	+ / +
	Follow up	K F			11.1	3	88	27	
3	Base line	R K	20	SS	9.6	10	79	24.9	+ / +
	Follow up	R K			13.5	3	91	30.7	

*In-vitro* cell culture studies will also be done where HU will be used at different concentrations to evaluate the F-cell response in the erythroid cell cultures. Hb analysis will be done by HPLC and F-cell distribution will be done by flow cytometry.

**Genetic valuation of 12 STR molecules among some primitive tribal population groups.**

*Year of Commencement* : 2002

*Year of Completion* : 2004

Microsatellites are Short Tandem Repeats (STR's) composed of a core unit of 1-5 bases. These elements are ubiquitously and uniformly dispersed throughout the eukaryotic genome and show high degree of polymorphism / heterozygosity and are considered to be highly informative markers in the various fields of modern genetics.

The objective of the present study was to determine the extend of genetic variation at twelve dinucleotide microsatellite markers in seven primitive tribal populations with a diverse ethnic linguistic and geographic background from Maharashtra, Gujarat and Tamilnadu. Some preliminary findings on allele frequencies were reported by us earlier and the study has now been completed. DNA samples of 185 untreated individuals were analysed for 12 STR loci (D12S83, D13S218, D12S78, D13S217, D12S1659, D13S285, D13S170, D12S1723, D13S175, D13S263, D12S1617, D12S346) of chromosome 12 and 13 by multiplex PCR

using commercially available ABI prism linkage mapping setS V 2.5 kits based on FAM, VIC and NED fluorescent labeled detection. The samples were run on ABI prism 310 genetic analyzer by using gene scan programme. Alleles at 12 loci have been designated by the size (in base pairs) of their PCR product.

The allele frequencies among the seven primitive tribal groups at 12 STR loci has already been reported. The number of alleles at each loci tested varies from 6 to 17 with an average of 11 at each locus and the maximum number of alleles are found in D12S78. In general the seven populations share the some common alleles and in majority of the cases, the most pre-dominant allele is the same with the variable allele frequencies. Some of the rare alleles are also observed and mainly present among the Madias in low frequencies.

Locus and average population heterozygosities based on allele frequencies alongwith  $G_{ST}$  values are given in Table 1. The average heterozygosity is uniformly high (0.707) in the studied population and varies from 0.705 to 0.794. Among the loci D13S263 show the highest levels of heterozygosity in the populations (0.643 – 0.923) and D 12S1659 the lowest (0.300 – 0.678). Hardy-Weinberg equilibrium analysis revealed that these populations are in genetic equilibrium at almost all the loci tested. The overall extent of gene differentiation among the seven groups is high ( $G_{ST}= 0.052$ ). However, there is considerable heterogeneity in the degree of differentiation at different loci while it high 10% in the case of D13S175, it is low at 2.6% in the case of D12S170.

The UPGMA tree constructed for the seven populations using the DA distances is presented in Fig 1. The UPGMA tree suggests three clusters of populations and Moolukurumbas from Nilgiri appear to be far apart from the other six populations. The clusters of the remaining 6 populations shows two different clusters constituting 1) Katkaris from Maharashtra, Kolcha and Kotvadia from Gujarat 2) Madia from Maharashtra Irula and Kurumba from Tamilnadu.

**Table 1. Locus, Average heterozygosities and  $G_{ST}$  values**

Locus	Katkari (n=64)	Madias (n=80)	Irulas (n=68)	Kurumbas (n=46)	Moolu Kurumbas (n=36)	Kolchas (n=44)	Kotvadias (n=32)	G <sub>ST</sub>
D12S83	0.750	0.865	0.793	0.650	0.714	0.894	0.538	0.042
D13S218	0.785	0.675	0.655	0.450	0.357	0.789	0.923	0.078
D12S78	0.928	0.838	0.793	0.900	0.929	0.894	0.846	0.044
D13S217	0.928	0.838	0.827	0.850	0.357	0.684	0.846	0.064
D12S1659	0.678	0.432	0.551	0.300	0.500	0.368	0.384	0.054
D13S285	0.857	0.757	0.862	0.800	0.990	0.789	0.846	0.035
D13S170	0.821	0.892	0.586	0.700	0.928	0.947	0.692	0.026
D12S1723	0.643	0.784	0.586	0.800	0.714	0.684	0.384	0.036
D13S175	0.607	0.649	0.689	0.500	0.571	0.684	0.538	0.101
D13S263	0.821	0.810	0.896	0.700	0.643	0.684	0.923	0.052
D12S1617	0.821	0.729	0.827	0.900	0.928	0.842	0.692	0.067
D12S346	0.893	0.838	0.862	0.950	0.857	0.789	0.846	0.042
Average	0.794	0.759	0.744	0.708	0.708	0.754	0.705	0.052

n = Number of chromosomes studied.

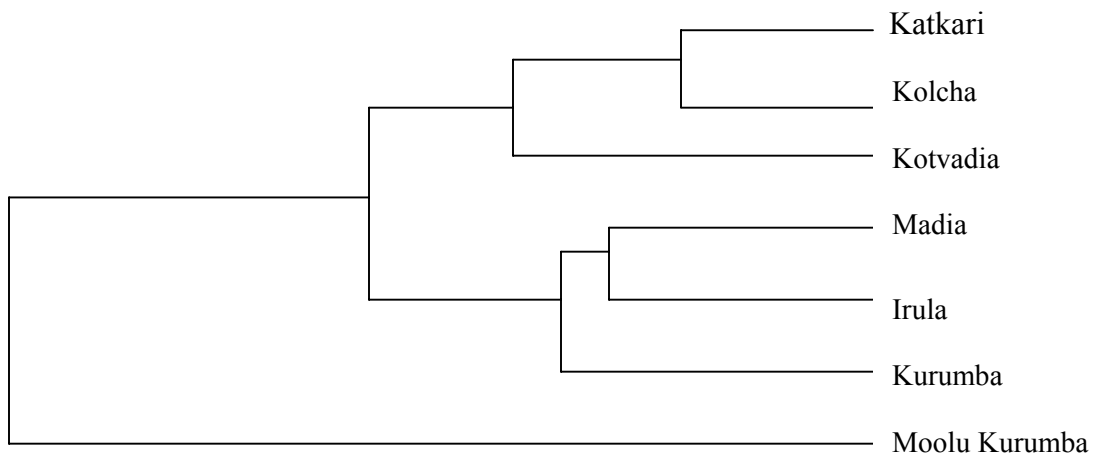


Figure 1: UPGMA tree depicting genomic affinities among seven primitive tribal populations of India

**Molecular characterization of  $\beta$  - thalassemia and 1<sup>st</sup> trimester prenatal diagnosis.**

*Year of commencement* : 1992

*Year of completion* : Ongoing

**a) Characterization of mutations in  $\beta$ -thalassemia and other abnormal hemoglobins.**

This project was continued and molecular characterization was undertaken in heterozygotes identified during population screening as well as families referred for confirmation of diagnosis as well as for prenatal diagnosis. Samples included individuals from ethnic groups and different regions in the country.

Mutation analysis was undertaken in 357 heterozygotes using a combination of methods like reverse dot blot hybridization and ARMS.

The following mutations were detected

IVSI – 5 (G $\rightarrow$ C)	151
619 bp deletion	22
Codons 819 (+G)	7
IVS 1 –1 (G $\rightarrow$ T)	13
Codons 41/42 (-CTTT)	8
Codon 15 (G $\rightarrow$ A)	34
Codon 30 (G $\rightarrow$ C)	4
Cap site +1 (A $\rightarrow$ C)	6

Codon 5 ( --CT)	5
Codon 16 ( --C)	2
IVS II-837 (T → G)	6
Codon 6 (GAG → GTG)Hb S	32
Codon 26 (GAG → AAG) Hb E	1
Un characterized	4

The mutations characterized in 51 homozygotes or double heretozygotes were as follows:

IVST – 5 (G→C) homozygous	20
Codons 819 (+G) homozygous	1
Codon 15 (G → A) homozygous	5
Codon 30 (G → C) homozygous	1
IVS II-837 (T →G) homozygous	2
IVS 1 –5 (G→ C) + Codon 30 (G→ C )	1
IVS 1 – 5 (G →C) + Hb E	3
IVS 1 –5 ( G→C) + Codons 819 (+G)	2
IVS 1--5( G→C)+ cap site +1 (A→C)	1
IVS 1--5( G→C)+ 619 bp del	1
IVS 1--5( G→C)+ Hb S	3
IVS 1--5( G→C)+ Codon 15 (G → A)	1
IVS 1--5( G→T) + 619 bp del	2
Codons 819 (+G) + IVS I-1 ( G→T)	1
IVS 1--5( G→C)+ Codon 5 (- CT)	1
IVS 1--5( G→C)+ Hb S	3
IVS 1--5( G→C)+ Codon 15 (G →A)	1
IVS 1--1( G→T)+ 619 bp del	2
Codons 819 (+G) + IVS 1--5( G→T)	1

IVS 1--5( G→C)+ Codon 5 (- CT)	1
Codon 15 (G → A) + cap site +1 (A→C)	1
Codon 16 (-C) + Hb E	1
IVS 1--5( G→C)+ ↑ Hb F	1
IVS 1--5( G→C)+ Hb D	1
619 bp del + Hb D	1
IVS 1 – 1 (G →T) + Hb Q	1

**b) First trimester prenatal diagnosis of thalassemia syndromes.**

Ninety couples were referred for first trimester prenatal diagnosis of thalassemia from different parts of the country. Among them, 73 were at risk of having a child with  $\beta$ -thalassemia major, 11 with sickle cell anemia, 4 with Hb S- thalassemia, 1 with Hb E- thalassemia, 1-with  $\delta\beta$  + thal  $-\beta$  thalassemia. Six couples had come prospectively before having an affected child.

CVS was done between 9.3 to 14.1 weeks gestation. The mutations were detected using combinations of reverse dot blot hybridization, ARMS and DGGE. With this strategy diagnosis was possible in 86 couples. In two couples where mutations could not be characterized, diagnosis was given using framework analysis.

2 couples were called for cordocentesis in the second trimester.

Twenty-six fetuses were found to be affected (22 with  $\beta$  -thalassemia major, 2 with sickle cell disease, 1 with Hb S thalassemia, 1 with Hb E thalassemia). These couples were given the option to terminate the pregnancies. One couple had a twin pregnancy. In this case one fetus was  $\beta$  - thalassemia heterozygote (IVS 1 -5 (G→C) and the other fetus was  $\beta$  - thalassemia homozygous. (IVS 1-5 (G→C) homozygous.

The mutations in the affected fetuses were as follows:

IVS1 – 5 (G →C) homozygous	- 9
Codon 15(G→A) - “ -	- 2

619 bp deletion homozygous	- 2
Hb S homozygous	- 2
IVS 1- 5 (G→C) + Hb S	- 1
IVS 1- 5 (G→C) + Codon 16 (-C)	- 1
IVS 1- 5 (G→C) + Codons 41/42 -(CITT)	- 2
IVS 1- 5 (G→C) + 619 bp del	- 1
IVS 1- 5 (G→C) + Hb E	- 1
IVS 1- 5 (G→C) + 619 bp del	- 3
Codons 41/42 (-CTTT) + Codons 15 (G→A)	- 1
Codons 41/42 (-CTTT) + 619 bp del	- 1

### **Second Trimester Prenatal Diagnosis of Hemoglobinopathies.**

Cordocentesis and fetal blood analysis was done in 34 couples who came late for prenatal diagnosis (18 to 22 weeks) or where both the parental mutations could not be identified.

Among these couples 28 were at risk for  $\beta$ -thal major, 4 for sickle cell anemia, one for Hb E thalassemia and one for Hb S D Disease. 5 fetuses were affected (Thal. Major – 3, sickle cell anemia – 1, Hb S D disease-1) were given the option to terminate the pregnancies.