GENETIC ANALYSIS IN THALASSAEMIA

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- Genetic Haemoglobin Disorders
- Molecular genetic techniques
- Genetic basis in Pakistan
- Clinical applications

Genetic Haemoglobin Disorders

- Heterogeneous
- Inherited
- <u>Thalassaemia</u>
  - Quantitative (reduced) production of globin
- <u>Abnormal Haemoglobins</u>
  - Qualitative defect (Hb-S, Hb-D)
  - Some may also have a quantitative defect (Hb-E)

Genetic Classification of Thalassaemia

α-Thalassaemias:

- α^o -Thalassaemias
- α^+ -Thalassaemias
  - Deletion
  - Non-deletion
- With α or β-chain Hb structural variants
- With β-Thalassaemia

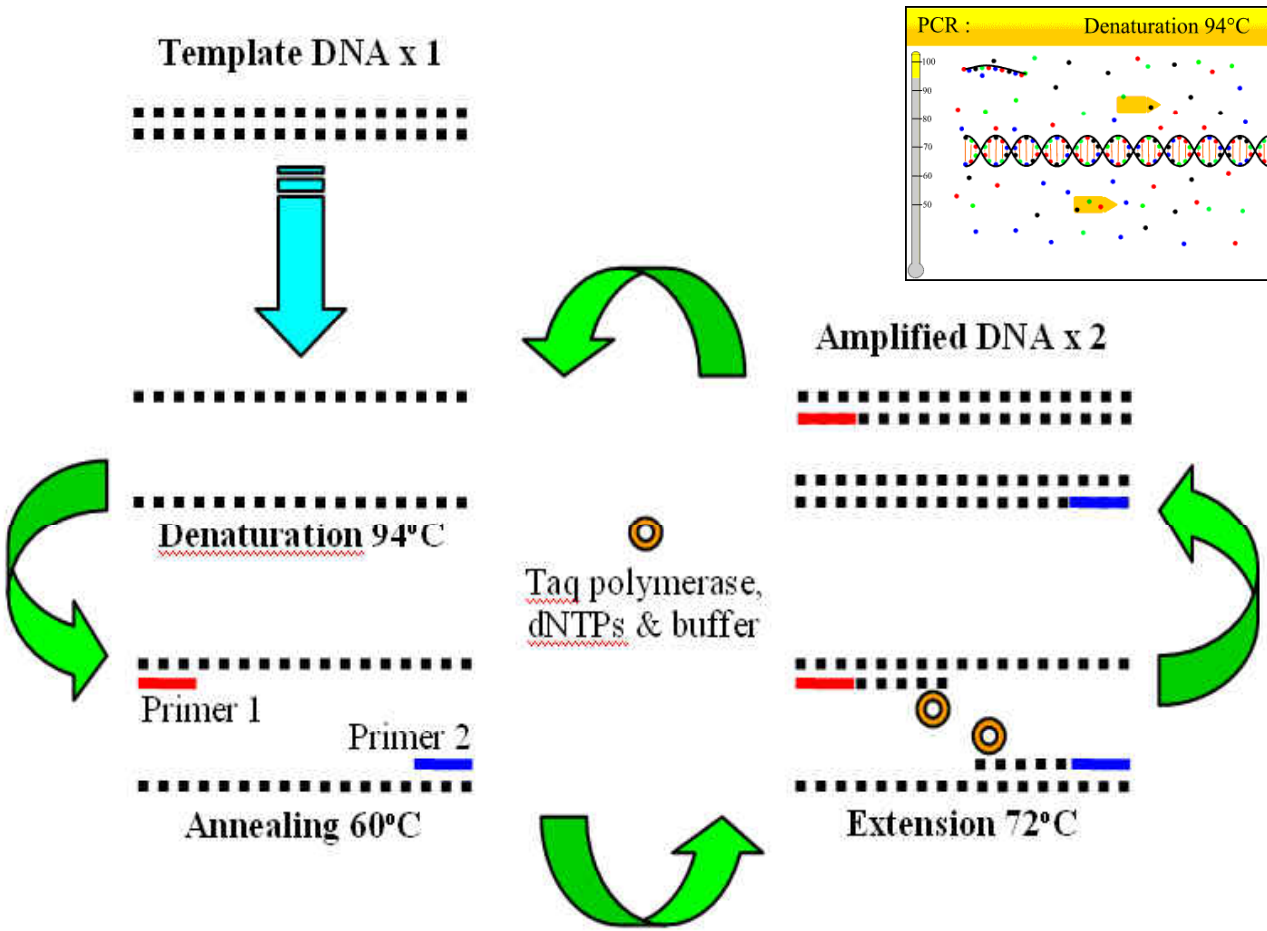
β-Thalassaemias:

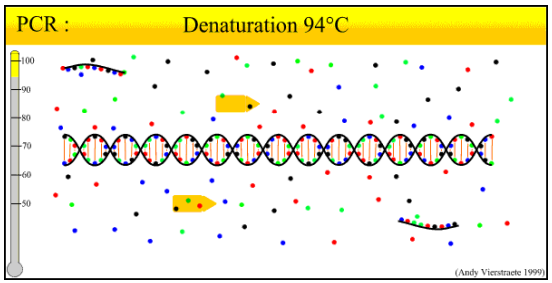
- β^o -Thalassaemias
- β^+ -Thalassaemias
- With α or β-chain Hb structural variants
- With α-Thalassaemia

$\delta\beta$-Thalassaemia:

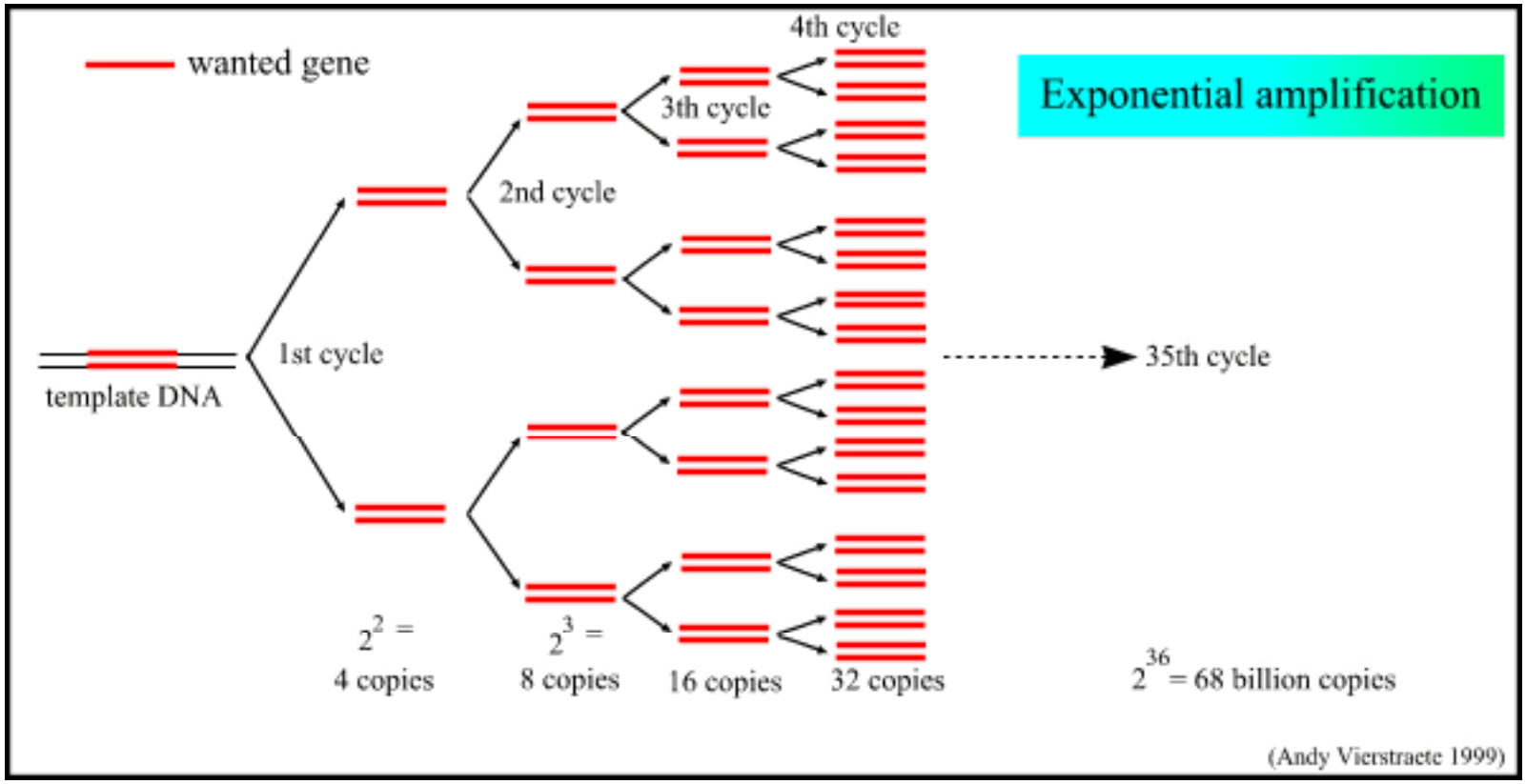
Hereditary Persistence of Fetal Haemoglobin (HPFH):

Polymerase Chain Reaction (PCR)

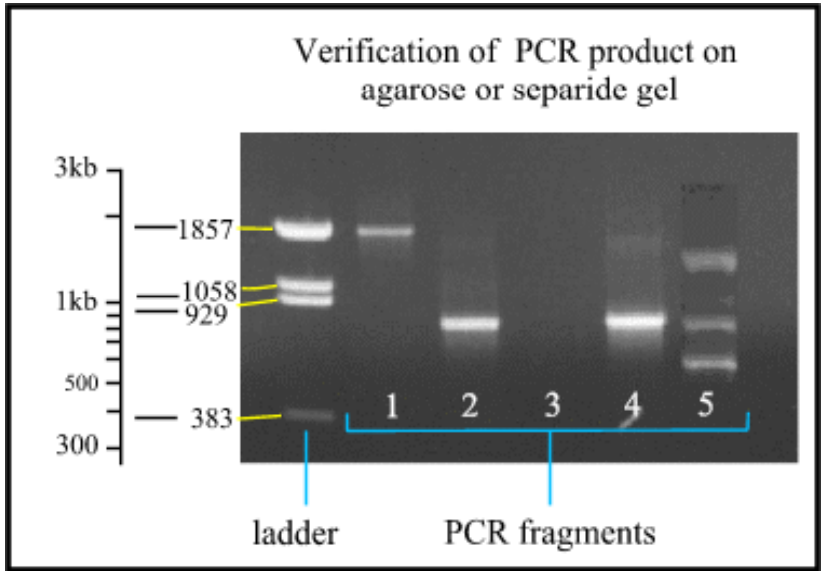




(S. Ahmed 2013)


wanted gene
Exponential amplification
4th cycle
3th cycle
2nd cycle
1st cycle
template DNA
35th cycle
2^2 =
4 copies
2^3 =
8 copies
16 copies
32 copies
2^{36} = 68 billion copies
(Andy Vierstraete 1999)


Clear


Verification of PCR product on
agarose or separide gel
3kb
1kb
500
300
1857
1058
929
383
1
2
3
4
5
ladder
PCR fragments

Molecular Genetic Methods

- Mutation Analysis
  - Specific methods
    - Amplification Refractory Mutation System (ARMS)
    - Dot Blot & Reverse Dot Blot
    - Sequencing
  - Non specific methods
    - DGGE
    - SSCP
- Linkage analysis

Amplification Refractory Mutation System (ARMS)

IVSI-5 (G-C)

```
A.      5′-GGCCCTGGGCAGGTTGCTATCAAGGTTACAAGACAGGTTTAAGGAG-3′
   Mutation primer  <--3′-GATTGTTCCAATGTTCTGTCCAAATTCCTC-5′

B.      5′-GGCCCTGGGCAGGTTGGTATCAAGGTTACAAGACAGGTTTAAGGAG-3′
   Mutation primer  <--3′-GATTGTTCCAATGTTCTGTCCAAATTCCTC-5′

C.      5′-GGCCCTGGGCAGGTTGGTATCAAGGTTACAAGACAGGTTTAAGGAG-3′
   Normal Primer    <--3′-CATTGTTCCAATGTTCTGTCCAAATTCCTC-5′

D.      5′-GGCCCTGGGCAGGTTGCTATCAAGGTTACAAGACAGGTTTAAGGAG-3′
   Normal Primer    <--3′-CATTGTTCCAATGTTCTGTCCAAATTCCTC-5′
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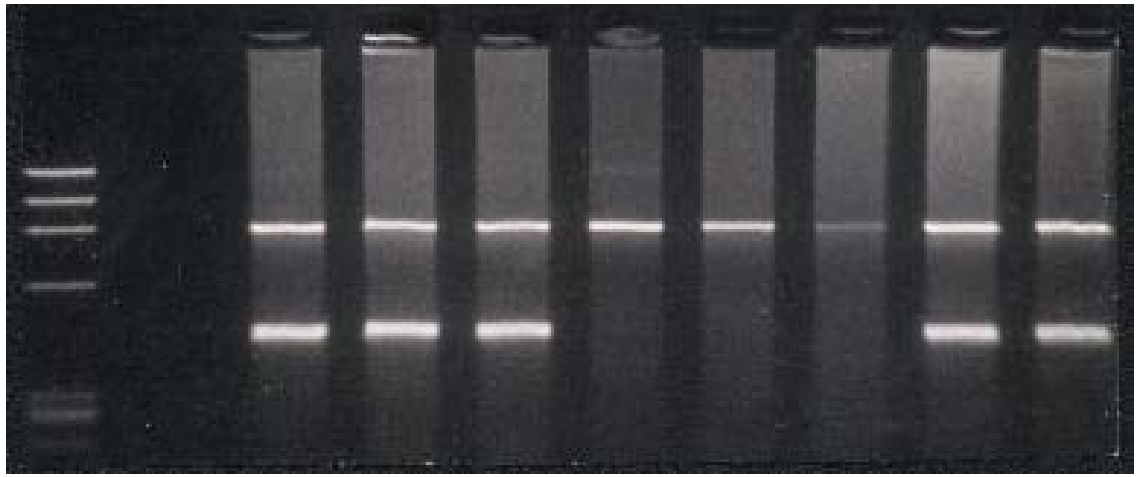
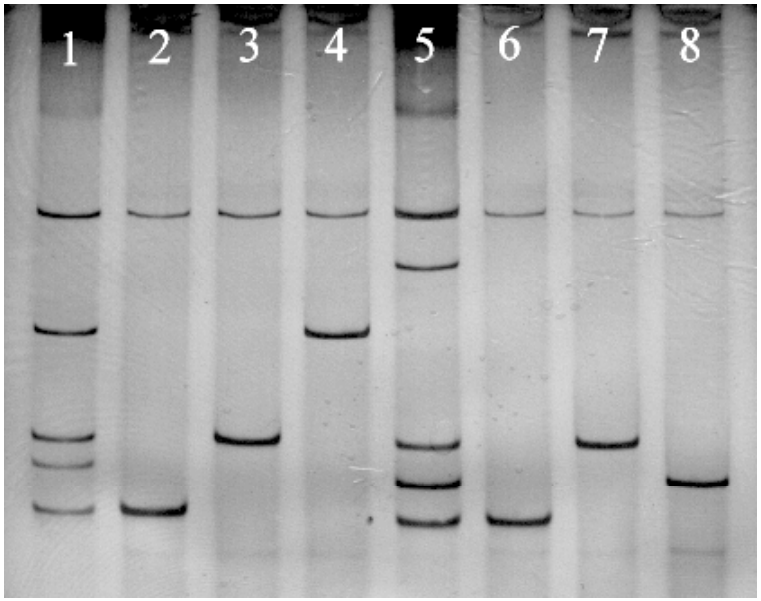
(S. Ahmed 2013)

Amplification Refractory Mutation System (ARMS)



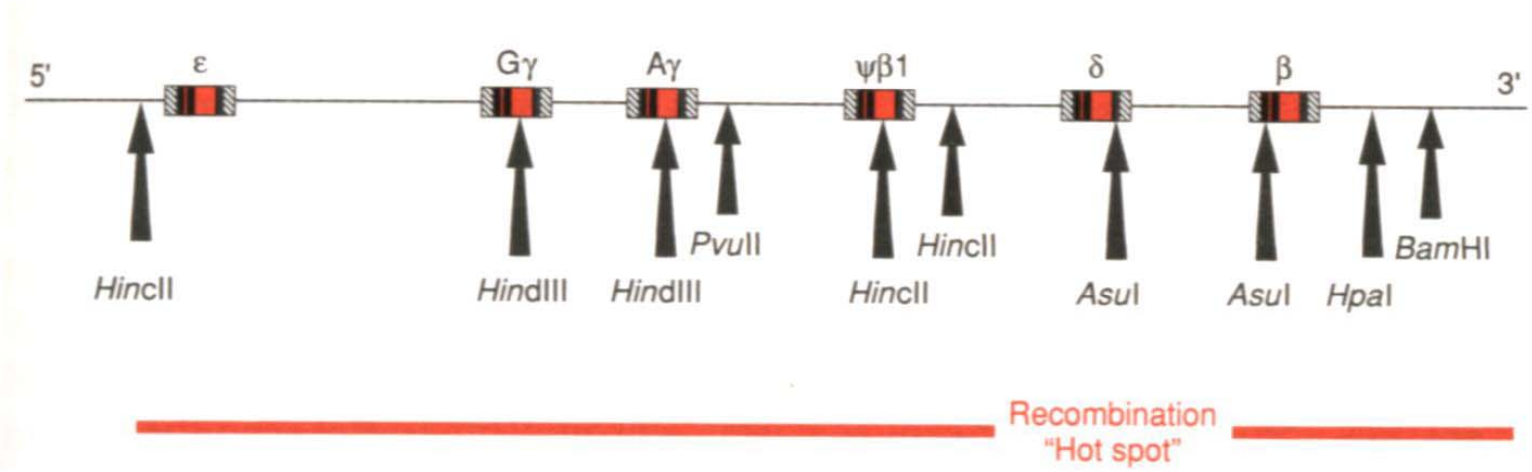
Multiplex ARMS PCR for Thalassaemia



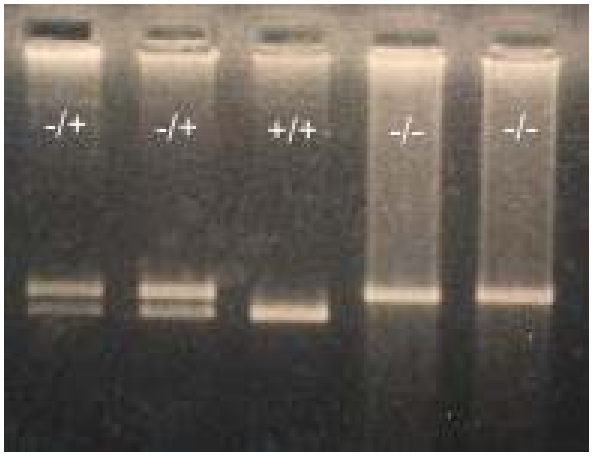
(S. Ahmed et al, 2000)

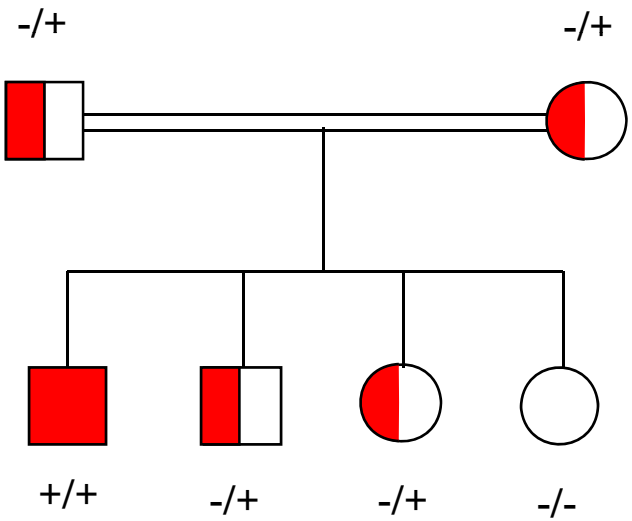
Primer ID:	Mutations Pooled:	Amplified Product size:
AD-1	Fr 8-9 (+G) IVSI-5 (G-C) Fr 41-42 (-TTCT) IVSI-1 (G-T) Del 619bp	215 bp 285 bp 439 bp 280 bp 242 bp
AD-2	Cd 5 (-CT) Fr 16 (-C) IVSI-1 (G-T) Cd 30 (G-C) Cd 30 (G-A) IVSII-1 (G-A)	205 bp 238 bp 280 bp 280 bp 280 bp 634 bp
AD-3	Cd 15 (G-A) Cap+1 (A-C)	500 bp 567 bp

Linkage Analysis



Linkage Analysis

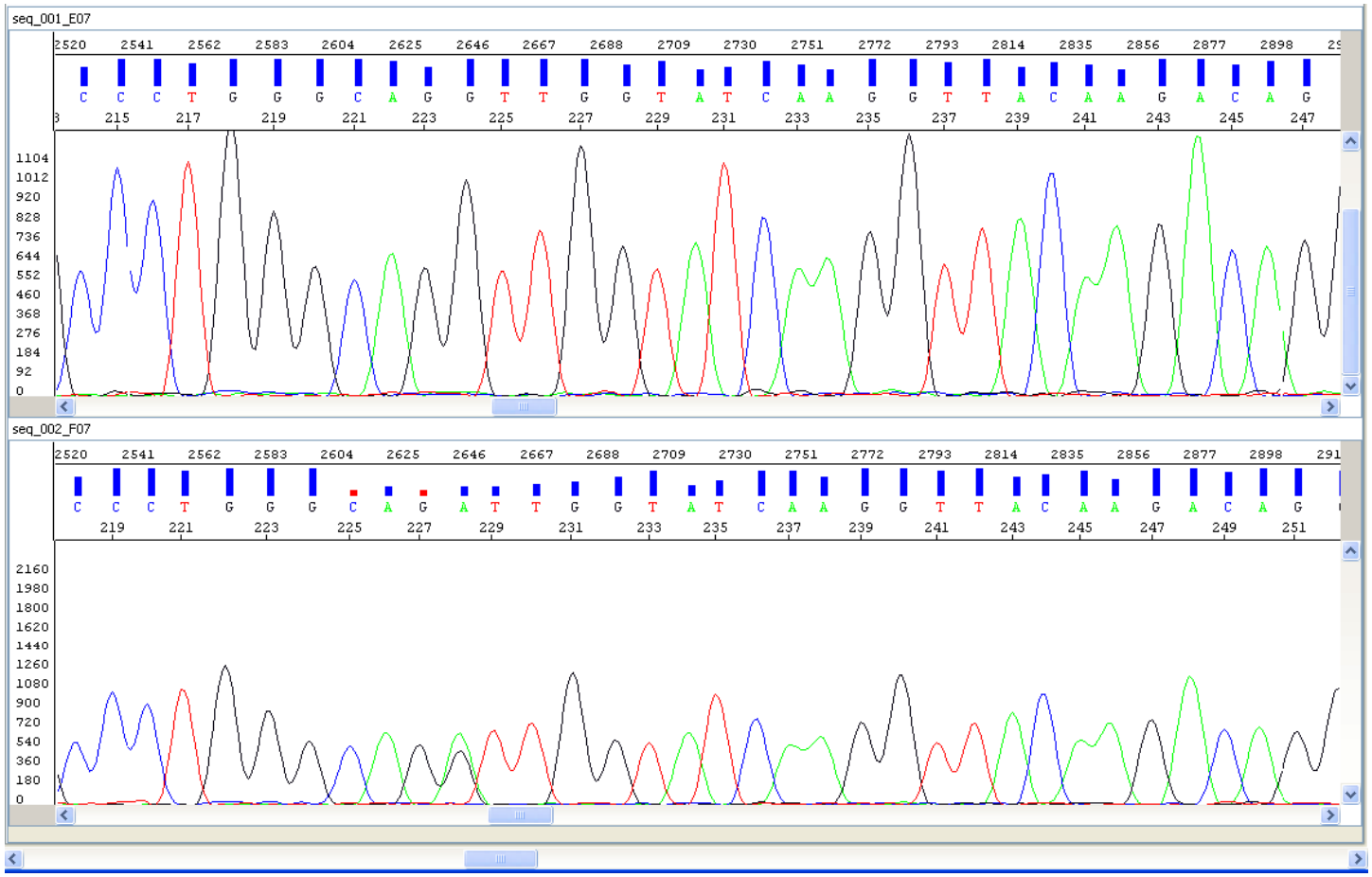




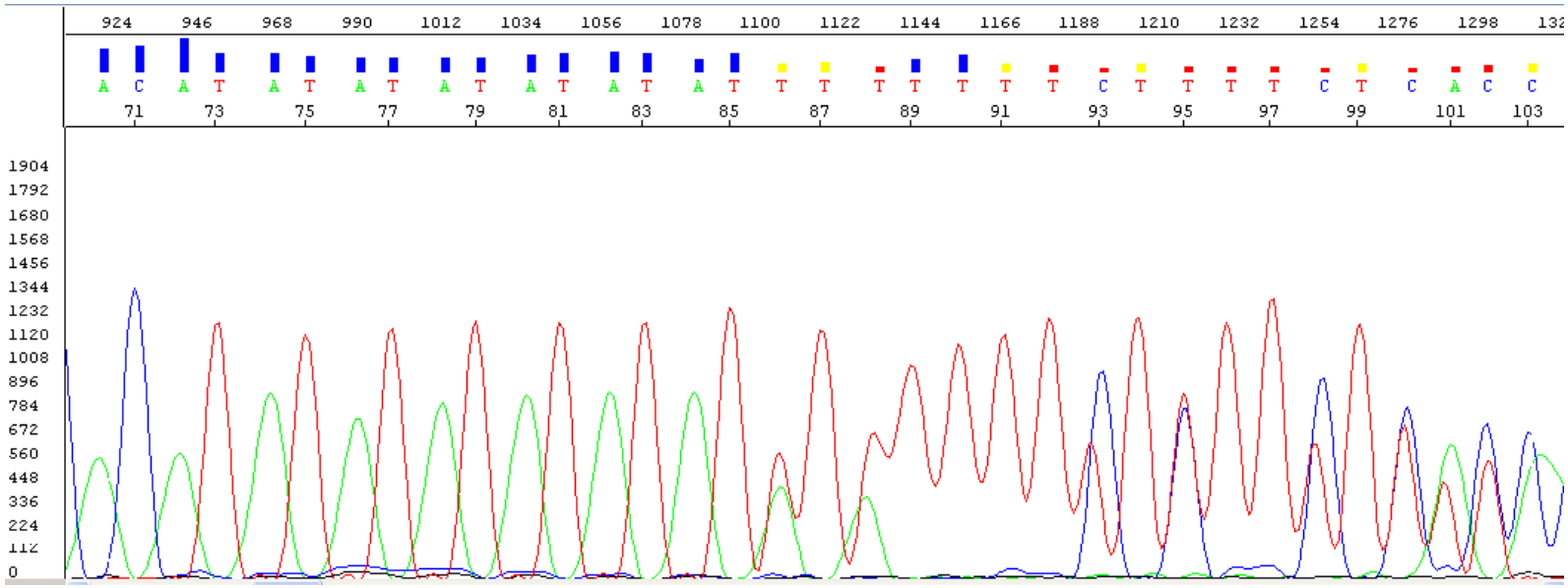
Mutation	Punjabi	Pathan	Sindhi	Baluchi	Mohajir	All
Common mutations						
IVSI-5 (G-C)	107 (27·2%)	27 (12·9%)	114 (43·9%)	131 (76·2%)	75 (41·4%)	454 (37·3%)
Fr 8–9 (+G)	146 (37·2%)	103 (49·1%)	29 (11·2%)	14 (8·1%)	23 (12·7%)	315 (25·9%)
Del 619 bp	14 (3·6%)	4 (1·9%)	36 (13·9%)	2 (1·2%)	29 (16·0%)	85 (7·0%)
Fr 41–42 (−TTCT)	36 (9·2%)	18 (8·6%)	16 (6·2%)	1 (0·6%)	11 (6·1%)	82 (6·7%)
IVSI-1 (G-T)	19 (4·8%)	4 (1·9%)	33 (12·7%)	2 (1·2%)	7 (3·9%)	65 (5.4%)
Uncommon mutations						
Cd 15 (G-A)	14 (3·6%)	13 (6·2%)	5 (1·9%)	9 (5·2%)	8 (4·4%)	49 (4·0%)
Cd 30 (G-C)	15 (3·8%)	1 (0·5%)	19 (7·3%)	3 (1·7%)	4 (2·2%)	42 (3·5%)
Cd 5 (−CT)	11 (2·8%)	16 (7·6%)	0 (0·0%)	1 (0·6%)	2 (1·1%)	30 (2·5%)
Fr 16 (−C)	6 (1·5%)	8 (3·8%)	6 (2·3%)	6 (3·5%)	3 (1·7%)	29 (2·4%)
Cap+1 (A-C)	9 (2·3%)	8 (3·8%)	0 (0·0%)	0 (0·0%)	3 (1·7%)	20 (1·6%)
Hb-E	3 (0·8%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	10 (5·5%)	13 (1·1%)
Cd 30 (G-A)	3 (0·8%)	2 (1·0%)	0 (0·0%)	2 (1·2%)	4 (2·2%)	11 (0·9%)
IVSII-1 (G-A)	6 (1·5%)	1 (0·5%)	0 (0·0%)	1 (0·6%)	2 (1·1%)	10 (0·8%)
Rare mutations						
−88 (C-T)	1 (0·3%)	2 (1·0%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	3 (0·3%)
IVSI-1 (G-A)	1 (0·3%)	0 (0·0%)	1 (0·4%)	0 (0·0%)	0 (0·0%)	2 (0·2%)
Fr 47–48 (+ATCT)	2 (0·5%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	2 (0·2%)
Fr 126–131 (−17 bp)	0 (0·0%)	2 (1·0%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	2 (0·2%)
Cd 39 (C-T)	0 (0·0%)	1 (0·5%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	1 (0·1%)
IVSI minus 25	0 (0·0%)	0 (0·0%)	1 (0·4%)	0 (0·0%)	0 (0·0%)	1 (0·1%)
Total	393 (100%)	210 (100%)	260 (100%)	172 (100%)	181 (100%)	1216 (100%)

(S. Ahmed et al, 1996)

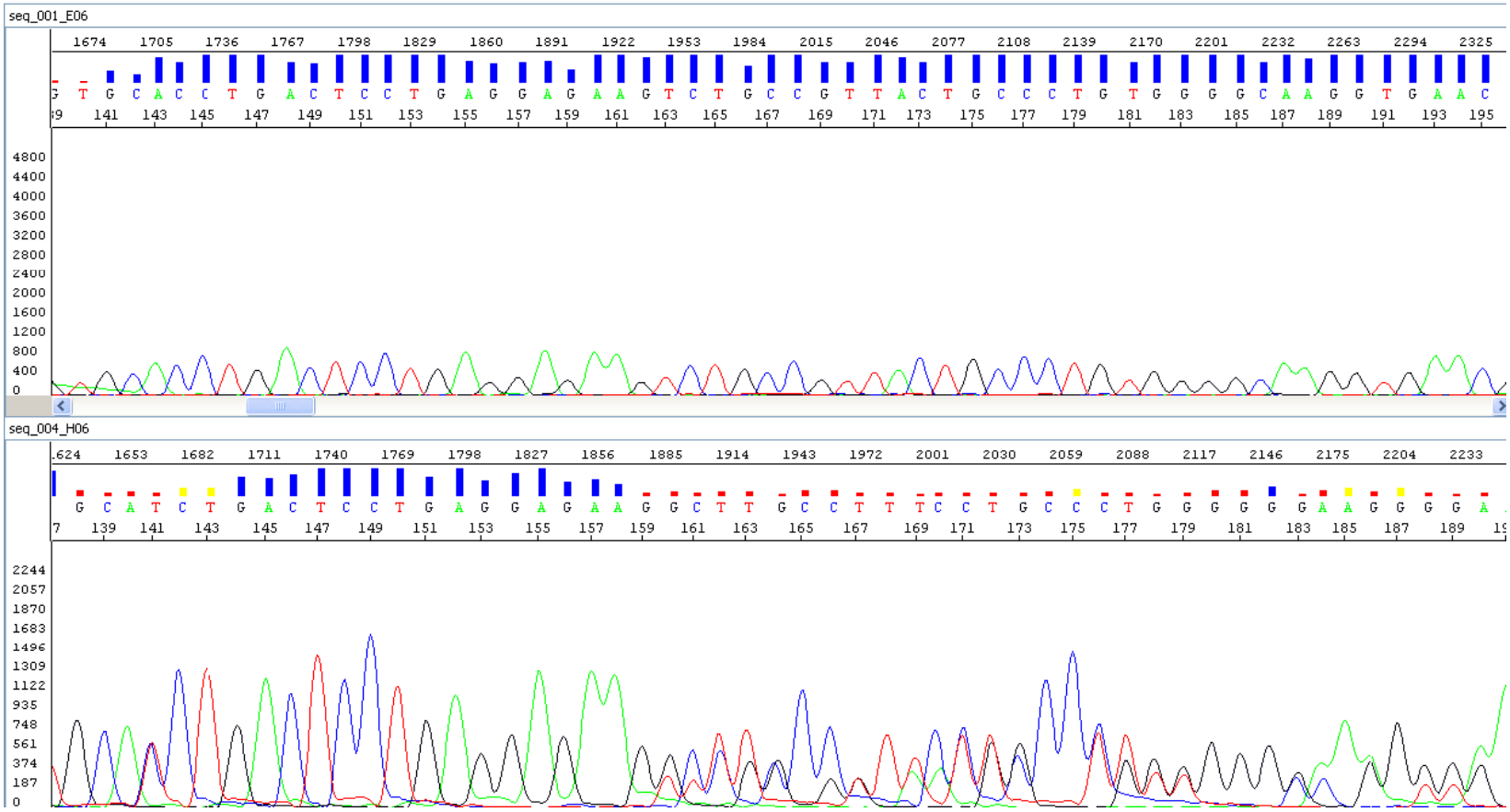
IVSI-1 (G-A)



-527 (+ATA)


924 946 968 990 1012 1034 1056 1078 1100 1122 1144 1166 1188 1210 1232 1254 1276 1298
A C A T A T A T A T A T A T A T A T T T T T T T T C T T T T C T C A C C
71 73 75 77 79 81 83 85 87 89 91 93 95 97 99 101 103
1904 1792 1680 1568 1456 1344 1232 1120 1008 896 784 672 560 448 336 224 112 0

Cd 45 (+T)

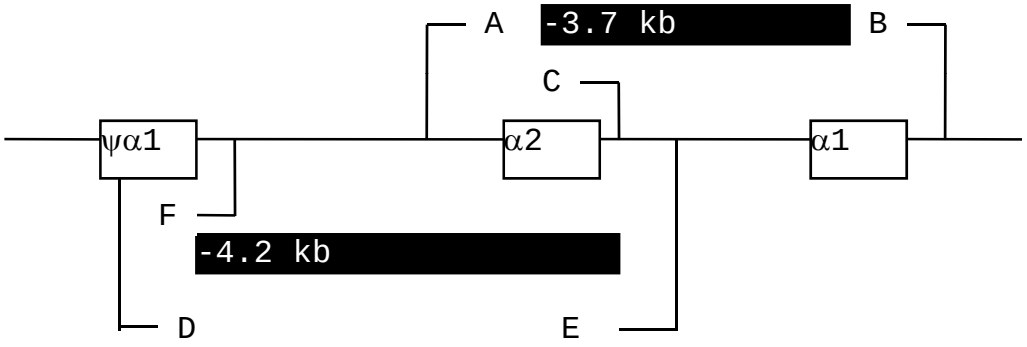


α-Thalassaemia in Pakistan

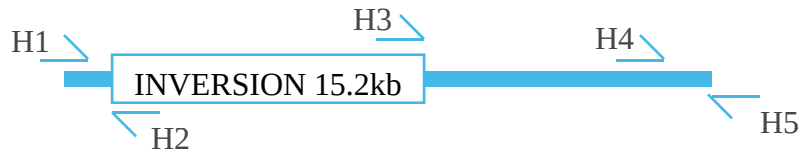
$-\alpha^{3.7}\ \alpha / \alpha\ \alpha$	8.3%
$-\alpha^{3.7}\ \alpha / -\alpha^{3.7}\ \alpha$	2.0%
$-\alpha^{4.2}\ \alpha / \alpha\ \alpha$	0.2%
Anti $-\alpha^{3.7}\ \alpha\ \alpha\ \alpha / \alpha\ \alpha$	0.9%
$-\alpha^{SEA}\ -\alpha^{SEA} / -\alpha^{3.7}\ \alpha$	?
Non deletional α-thal	?

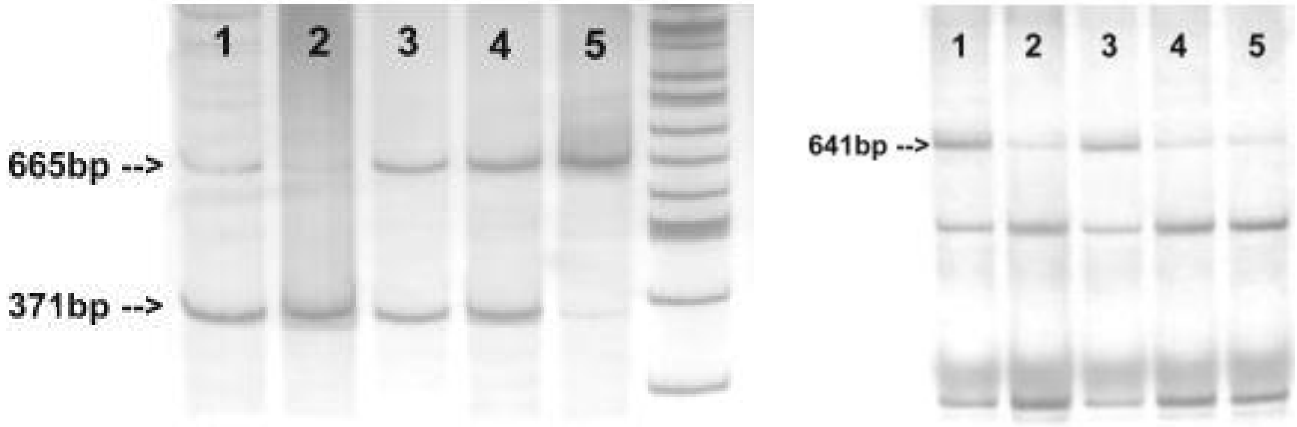
(S. Ahmed 1998; S. Khan et al, 2003)

PCR for α-Thalassaemia



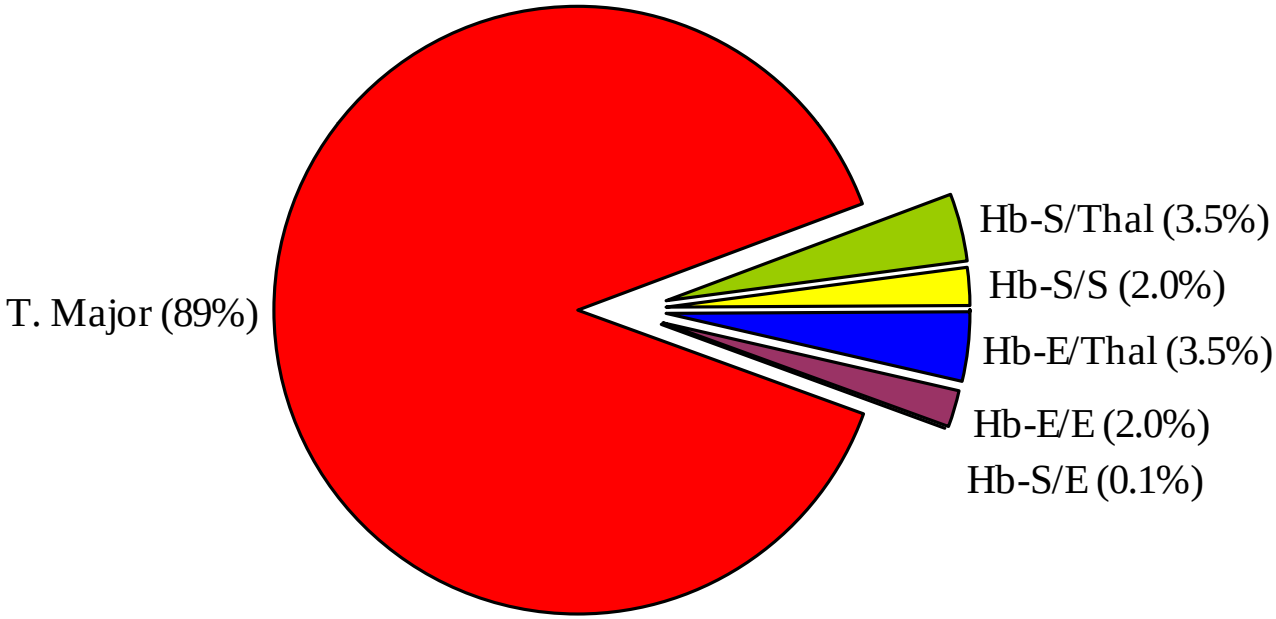
δβ-Thalassaemia in Pakistan
Inv/Del $^{G}\gamma(^{A}\gamma\delta\beta)^{o}$





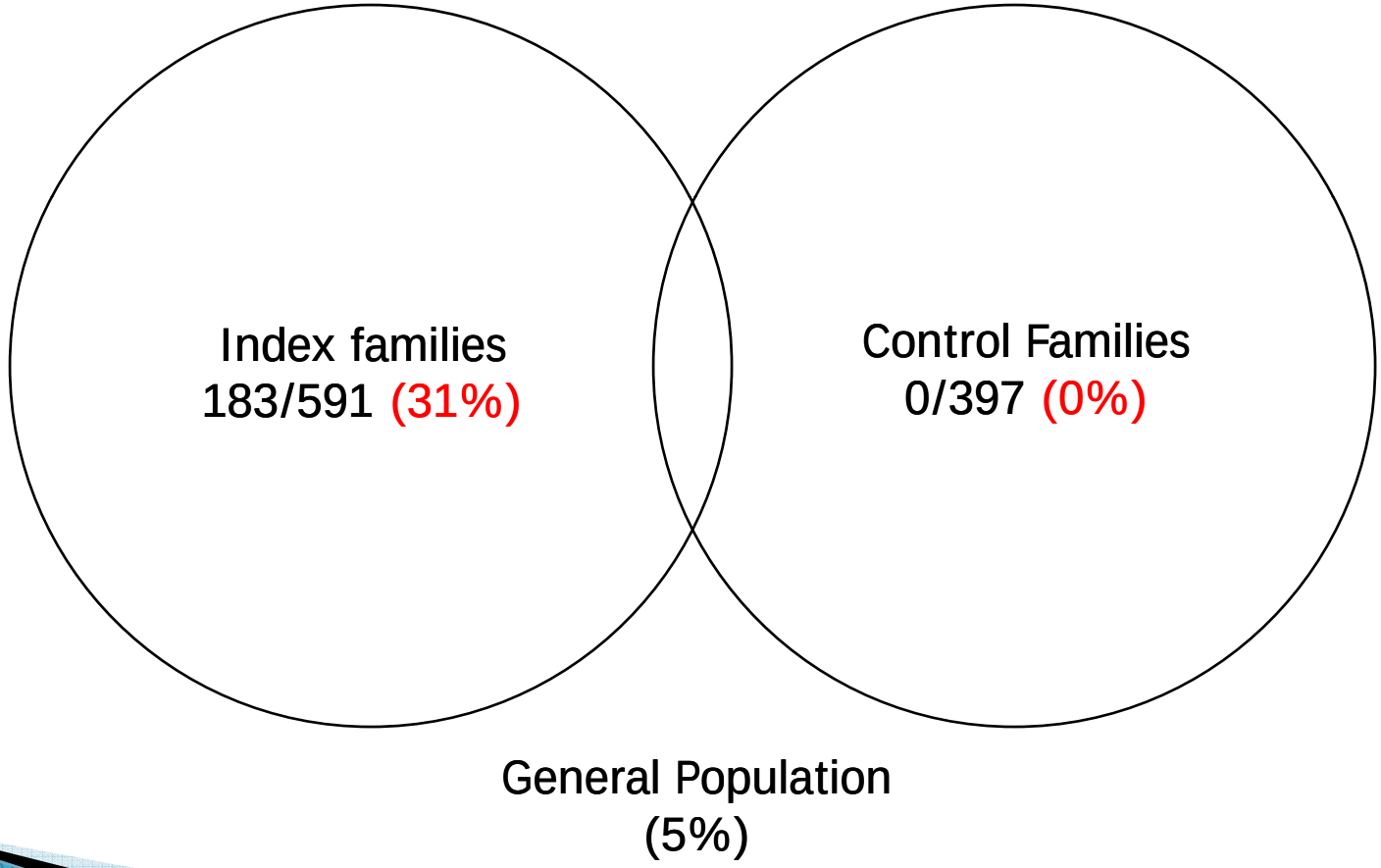
(S. Ahmed and M. Anwar, 2005)

Abnormal Haemoglobins in Pakistan



(S. Ahmed 1998)

Distribution of Thalassaemia Genes

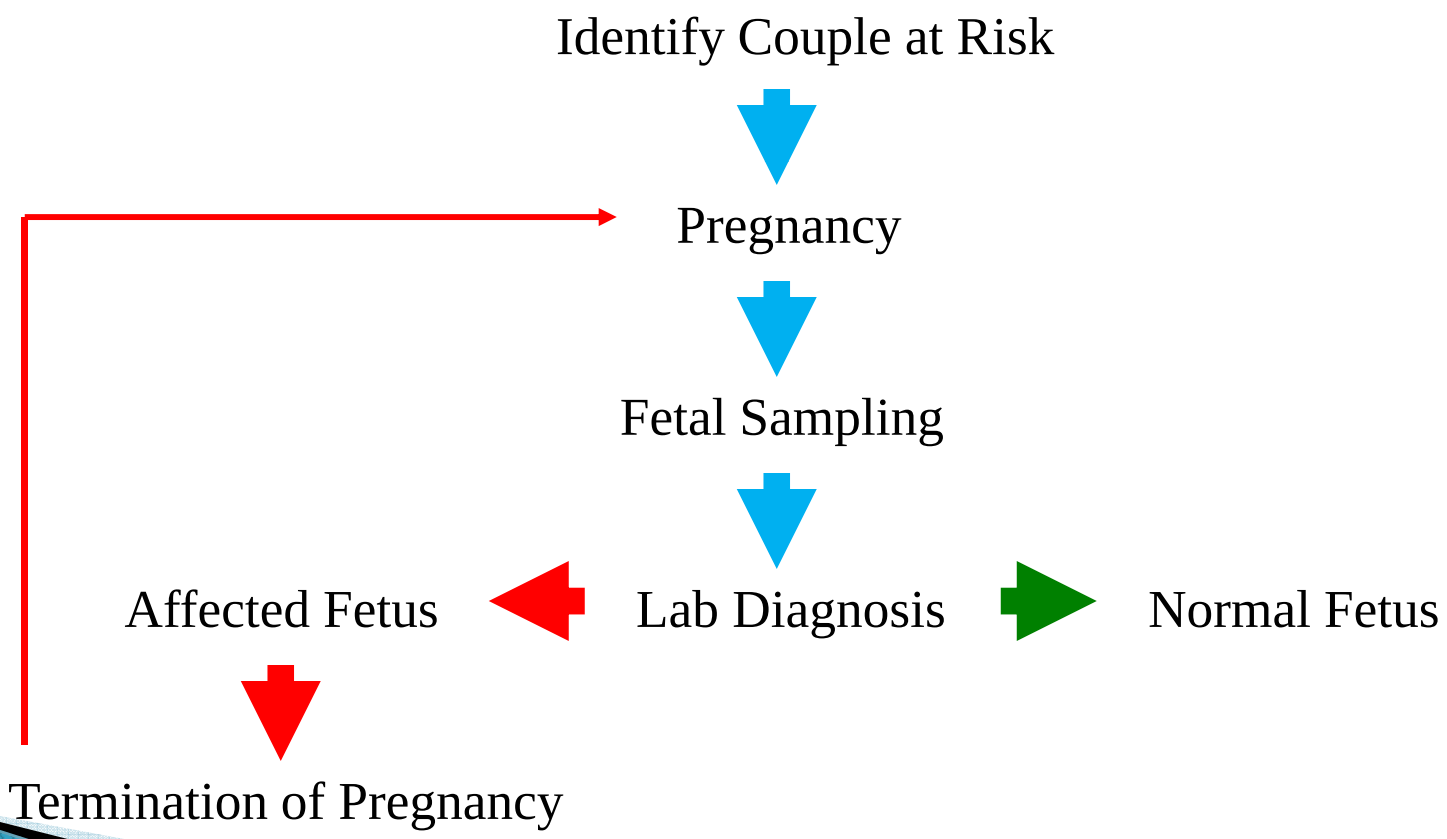


(S. Ahmed et al, 2002)

Clinical Applications

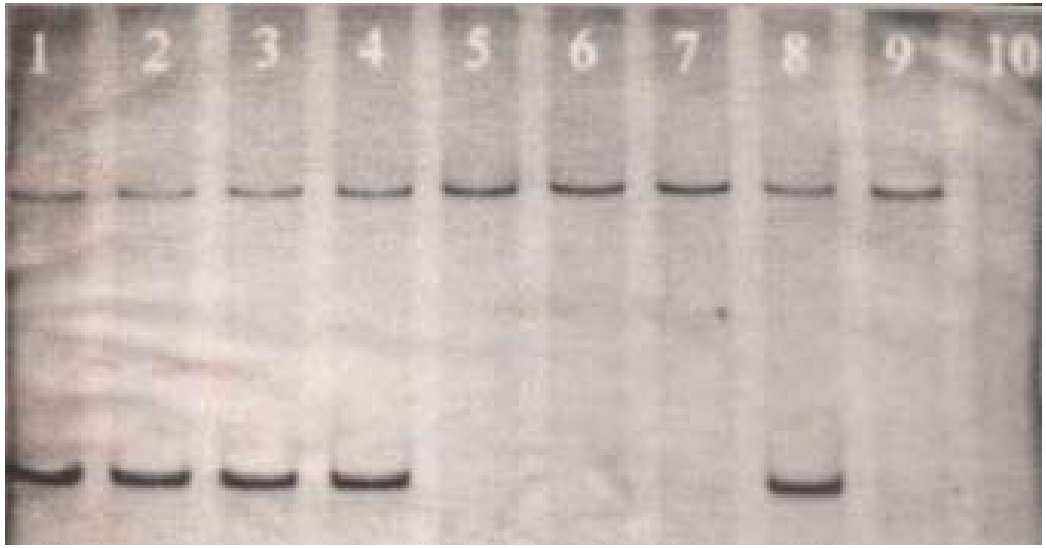
- Prenatal Diagnosis
- Diagnosis in previously transfused patients
- Silent thalassaemia alleles
- Distinction between structural variants
- Thalassaemia intermedia
- α-thalassaemia
- β-Thalassaemia carriers in certain situations
- Rare thalassaemias

Prenatal Diagnosis



Prenatal Diagnosis by ARMS

- <u>Mutation</u>
  1. Father
  2. Mother
  3. CVS
  4. CVS
  5. -ve
- <u>Normal</u>
  6. CVS
  7. CVS
  8. +ve
  9. -ve
  10. Blank

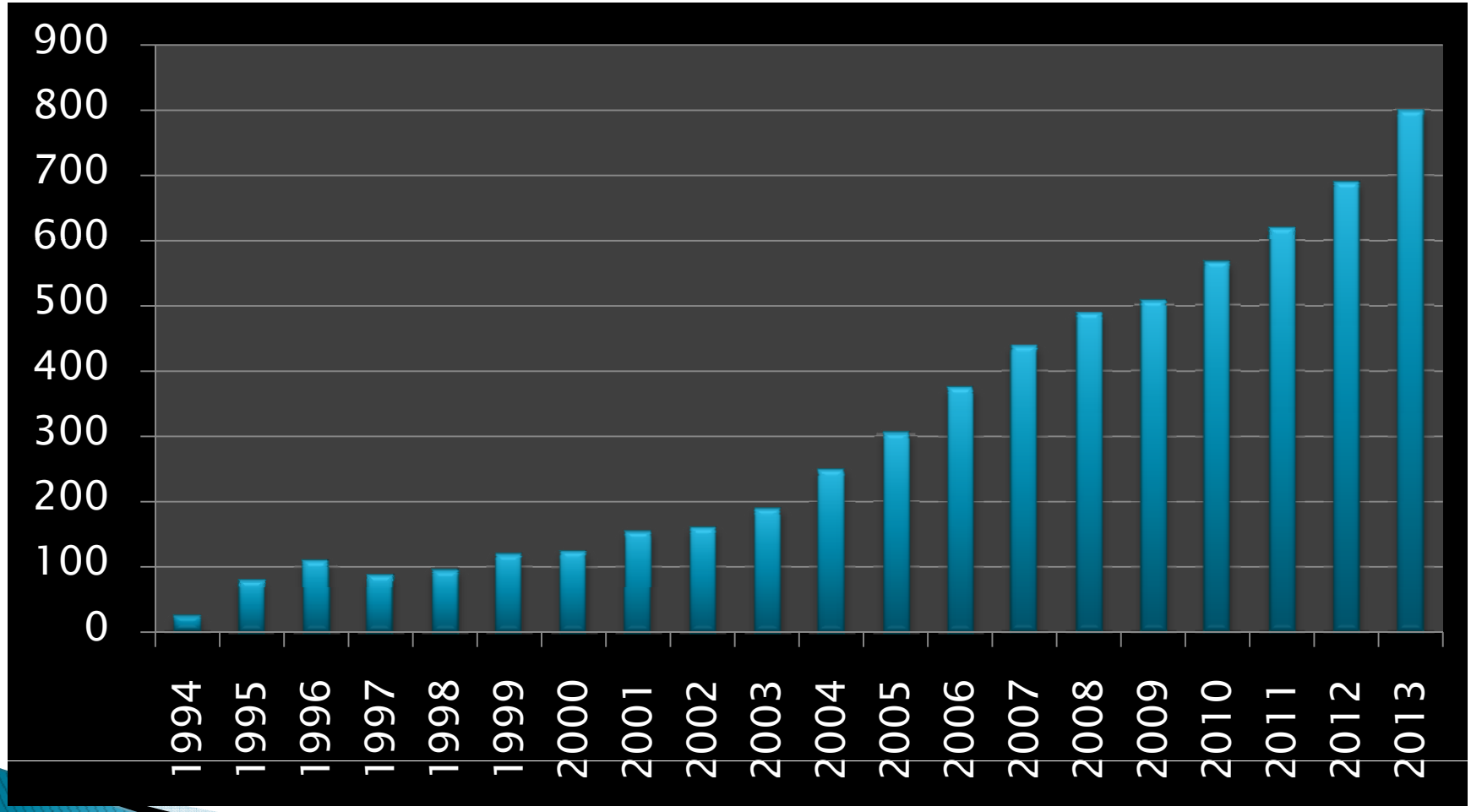


Misdiagnosis in PND (<0.5%)

- Maternal Contamination in Fetal Sample
- PCR Failure
- Clerical Mistakes
- Meotic Crossover in linkage analysis
- Non paternity

(S. Ahmed 2007)

PND for Thalassaemia in Pakistan
Armed Forces Institute of Pathology (AFIP)
Genetic Resource Centre (GRC)



Thalassaemia Intermedia

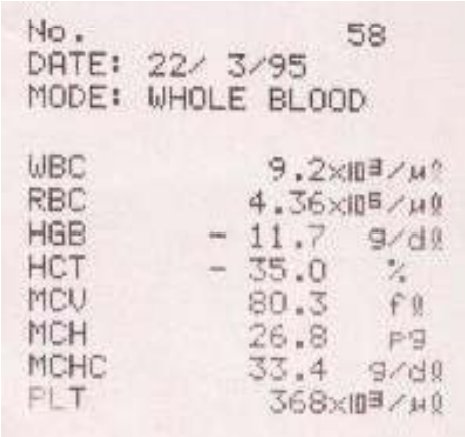
- Mild/Silent alleles
- Co-incidental α-thalassaemia trait
- Co-incidental structural variants
- Xmn-I polymorphism
- δβ-Thalassaemia

Thalassaemia Intermedia in Pakistan

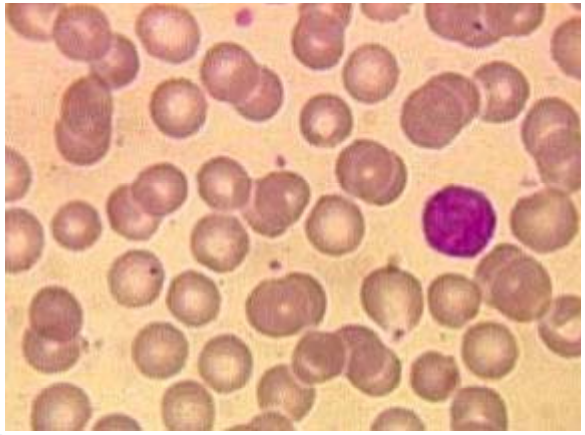
Cause of Thalassaemia Intermedia:	n (%):	Mean age:	
		At 1st transfusion:	At Examination:
Xmn-I +/+ genotype	14 36%)	6 years	13 years
β^+-mutation	6 (15%)	3 years	8 years
β^+-mutation and coincidental α-thalassaemia	6 (15%)	11¼ years	18 years
Unidentified thalassaemia mutation	2 (6%)	7½ years	12½ years
Coincidental α-thalassaemia	11 (28%)	9½ years	13½ years
Total	39	7 years	14 years

(S. Ahmed 1998)

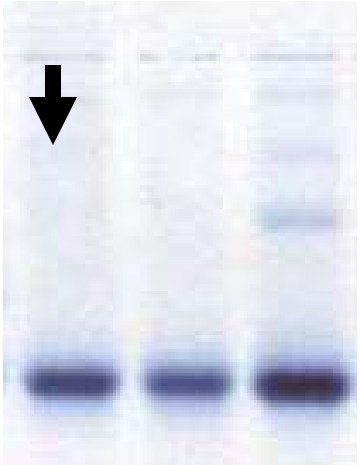
Silent β-thalassaemia Trait



No.	58	
DATE:	22/ 3/95	
MODE:	WHOLE BLOOD	
WBC	9.2×10³/μℓ	
RBC	4.36×10⁶/μℓ	
HGB	- 11.7	g/dℓ
HCT	- 35.0	%
MCV	80.3	fℓ
MCH	26.8	pg
MCHC	33.4	g/dℓ
PLT	368×10³/μℓ	



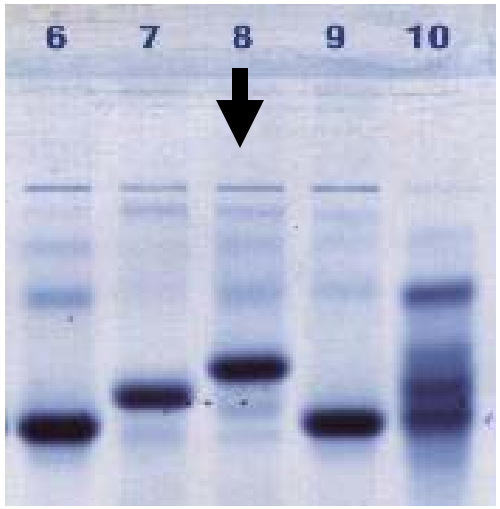
1 2 3



PCR: Cap+1 mutation

β-Thalassaemia Trait and Interacting Structural Hb Variants

No.		82
Date	24/ 6/02 13:01	
Mode	Whole Blood	
WBC	7.2	x10³/μL
RBC	5.88	x10⁶/μL
HGB	9.9	g/dL
HCT	33.3	%
MCV	56.6	fL
MCH	16.8	pg
MCHC	29.7	g/dL
PLT	306	x10³/μL



Hb-D/β-thalassaemia Trait