3 Materials and methods

Sequence and the time frame of the work:

The work in this study was done at the Department of Haematology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan and the Perinatal Centre, Department of Obstetrics and Gynaecology, University College Medical School, London. Table 3.1 gives a summary of the events, in a chronological order.

Haematological Methods:

Red cell indices:

The basic haematological parameters evaluated include, Haemoglobin (Hb) estimation, Total Red Blood Cell Count (TRBC), Mean Cell Volume (MCV), and Mean Cell Haemoglobin (MCH). These parameters were measured on blood collected in EDTA. An automated cell counter Sysmex K-1000 was used. However, Coulter S880 was also available as a backup support. Both instruments work on the principle of the electronic resistance method (Dacie and Lewis 1991). The instruments were calibrated according to the manufacturers instructions using the high, low and normal whole blood controls. Once calibrated, the performance of the instruments was regularly checked after every 20 samples by running a control.

Haemoglobin electrophoresis and Hb-A_2 estimation:

Blood collected in EDTA was thrice washed in normal saline (9.0 g/L NaCl). The washed red cells were lysed by adding two volumes of water and one volume of carbon tetrachloride (CCl_4). Clear haemolysate was separated after shaking and centrifugation at 1200 g for 10-20 minutes (Dacie and Lewis 1991). In a simple alternative method haemolysate was prepared by adding an equal volume of 1% saponin to the washed red cells. The final concentration of the haemolysate was adjusted to around 10 g/dl.

Table: 3.1. A chronological summary of the work carried out in the study.

Sr. No:	Event:	Place:	Period:
1.	Collection /extraction of DNA samples from the northern areas of Pakistan.	AFIP, Rawalpindi.	June 1992 to July 1993.
2.	Gene analysis of the DNA samples by ARMS method.	Perinatal Centre, UCL London.	July 1993 to January 1994.
3.	Establishment of facilities for gene analysis by PCR at AFIP Rawalpindi.	AFIP, Rawalpindi.	February 1994.
4.	Collection/extraction of DNA samples from southern areas of Pakistan and their mutation analysis by ARMS.	AFIP, Rawalpindi.	February 1994 to May 1994.
5.	Start of prenatal diagnostic service for thalassaemia in Pakistan.	AFIP, Rawalpindi.	May 1994.
6.	Thalassaemia Carrier screening in Punjabis and Pathans.	AFIP, Rawalpindi.	May 1994 to August 1994.
7.	Thalassaemia carrier screening in pregnant females.	AFIP, Rawalpindi.	August 1994 to September 1994.
8.	Thalassaemia carrier screening and collection of DNA samples in two large thalassaemic families.	AFIP, Rawalpindi.	October 1994 to December 1994.
9.	Gene analysis by DGGE.	Perinatal Centre, UCL London.	July 1995.
10.	Genomic sequencing of uncharacterized alleles.	Perinatal Centre, UCL London.	August 1995 to September 1995.
11.	Short Tandem Repeat (STR) analysis on DNA samples from the two families and controls.	Perinatal Centre, UCL London.	October 1995 to December 1995.
12.	Thalassaemia carrier screening on the extended families.	AFIP, Rawalpindi.	January 1995 to February 1995
13.	Thalassaemia carrier screening in Baluchi, Sindhi and Mohajirs.	AFIP, Rawalpindi.	March 1995
14.	Gene analysis for α-thalassaemia.	AFIP, Rawalpindi.	April 1995
15.	Assessment of the response of thalassaemic families to the availability of prenatal diagnosis.	Fatimid Thalassaemia Centre, Lahore.	May 1995

Haemoglobin electrophoresis was carried out on cellulose acetate membranes at alkaline pH (Dacie and Lewis 1991). At alkaline pH haemoglobin is a negatively charged protein and in an electrical field moves towards the anode (+). Most structural forms of haemoglobin can be separated due to a difference in the amount of charge on their surface.

The electrophoresis was carried out in a buffer containing Tris/EDTA/borate at pH 8.5. The cellulose acetate strips (Shandon UK) were allowed to soak in the buffer for at least 10 minutes. The haemolysate was applied to the cathode end of the strip. Electrophoresis was carried out at 200 V for 30 minutes or until adequate separation was achieved. The strips were stained in Ponceau-S and were subsequently dried at $37^{o}C$ for 30 minutes. With each batch of samples haemolysates prepared from cord blood and known cases of β-thalassaemia trait were included as controls.

$Hb\text{-}A_2$ was separated electrophoretically on cellulose acetate at pH 8.9 after applying 5-10μl of the haemolysate. The bands of Hb-A and $Hb\text{-}A_2$ were carefully cut and placed in 15 ml and 1.5 ml respectively of the buffer. Elution of the bands was allowed for 20-30 minutes. Absorbance of each fraction was measured at 413 nm against the buffer as blank. Percentage of $Hb\text{-}A_2$ was calculated as follows:

$$\%Hb\text{-}A_2 = \frac{A^{413}\ Hb\text{-}A_2}{(10 \times A^{413}\ Hb\text{-}A) + A^{413}\ Hb\text{-}A_2} \times 100$$

Microcolumn chromatography kits (Bio-rad, USA) were used on some samples to crosscheck the results of $Hb\text{-}A_2$. Commercial controls of $Hb\text{-}A_2$ (Bio-rad, USA) were used to check the quality control.

Strategy for carrier screening:

β-thalassaemia:

Haematological parameters including Hb, TRBC, MCV and MCH were recorded. $Hb\text{-}A_2$ estimation was done on all those who had MCV ≤75 fl or MCH ≤25pg (Modell and Berdoukas 1984). $Hb\text{-}A_2$ ≥3.5% confirmed the diagnosis of β-thalassaemia trait (Steinberg and Adams III 1991). In subjects who had $Hb\text{-}A_2$ in the borderline range (3-

3.4%) or who had Hb <9g/dl and Hb-A_2 in the normal range, multiplex ARMS PCR was carried out to screen for the thalassaemia mutations (Cao et al, 1994).

Abnormal haemoglobins:

Screening for abnormal haemoglobins by electrophoresis was possible only in cases that had MCV ≤75 fl or MCH ≤25pg.

α-thalassaemia:

α-thalassaemia screening was done by the PCR based detection of $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions (Baysal and Huisman 1994).

Gene analysis:

DNA extraction:

For gene analysis 5ml of blood was collected in EDTA. The red cells were lysed by 1% solution of triton X-100 and the white cells were lysed by 0.5 ml of 2% SDS solution containing 50 mM Tris and 20 mM Na_2EDTA (pH 8.0). Protein digestion was done with 250 µg Proteinase-K (Sigma, USA) at 37°C overnight or at 56°C for 2 hours. DNA extraction was done by phenol chloroform method (Maniatis 1989). The extraction was done in 1.5 ml Eppendorf tube by adding 250µl each of buffered phenol containing 0.1% 8-hydroxyquinolone (pH 8.0) and chloroform. Phenol chloroform extraction was done twice and was then followed by one wash in chloroform. Final precipitation of DNA was carried out in 70% ethanol. The extracted DNA was dried and re-dissolved in water to yield a final concentration of approximately 500ng/µl. The DNA samples were stored at -20°C until analysis.

Mutation analysis:

Strategy for mutation analysis:

The samples were first screened by the Amplification Refractory Mutation System (ARMS) for 15 β-thalassaemia mutations previously reported in Pakistani and Indian subjects (Varawalla et al, 1991a and 1991b). Those who were not positive for these mutations were screened by Denaturing Gradient Gel Electrophoresis (DGGE) as this

identifies the region of the gene where the mutation may be present (Cai and Kan 1990). The region of β-globin gene that was found by DGGE to have a mutation was sequenced by the di-deoxy chain termination method (Sanger et al, 1977). In samples where a mutation was not found by ARMS or an abnormal fragment by DGGE, had the entire β-globin gene sequenced with its immediate 5' and 3' flanking sequences.

Amplification Refractory Mutation System (ARMS):

The β-globin mutations screened for, and the sequence of the ARMS primers used is presented in Table: 3.2. The primers were in use for prenatal diagnosis of β-thalassaemia at the Perinatal Centre, UCH, London. Most of the primers were used in combination with an upstream (5') primer (No 3 in Table: 3.2). However, for Cd 15 (G-A) and Cap+1 (A-C) a downstream (3') primer (No 4 in Table: 3.2) was used. Another set of primers (No 1 and 2 in Table: 3.2) was used to amplify an 861 bp fragment of the distal part of the β-globin gene, to serve as an internal control for the PCR. The same set of primers was also used to screen for the 619 bp deletion, which when present results in amplification of a 242 bp fragment instead of the usual 861 bp fragment. Patients with homozygous β-thalassaemia who were positive for a mutation were then tested for the presence of the normal allele to test if they were homozygous for the mutation or not. Primers for the normal sequence (Table: 3.3) were used to identify the normal allele. Subjects with the normal allele were then tested for the presence of other mutations.

PCR conditions for ARMS:

The PCR for ARMS was carried out in a 25μl reaction mixture containing 5 pM of each primer, 0.5 units of Taq polymerase (Advanced Biotechnologies, UK), 30 μM of each dNTP (Advanced Biotechnologies, UK), 10 mM Tris HCl (pH 8.3), 50 mM KCL, 1.5 mM $MgCl_2$, 100 mg/ml gelatin (Sigma, UK), and 0.3-0.5 μg of genomic DNA. Primers were synthesized by Oswell UK, and Pharmacia Biotech, UK. Thermal cycling was carried out in an automated DNA thermal cycler (TC-480) from Perkin Elmer, USA. The regimen consisted of 25 cycles each consisting of denaturation at 94°C for 1 minute, primer annealing at 65°C for 1 minute, and DNA extension at 72°C for 1½ minutes. In the final cycle, the extension reaction was prolonged for another 3 minutes.

Table: 3.2. The mutations screened for and the sequence of the ARMS primers used.

Mutation	Primer	Used with	Size
IVSI-5 (G-C)	CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG	3	285
Fr 8-9 (+G)	CCT TGC CCC ACA GGG CAG TAA CGG CAC ACC	3	215
IVSI-1 (G-T)	TTA AAC CTG TCT TGT AAC CTT GAT ACG AAA	3	281
Fr 41-42 (-TTCT)	GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT	3	439
Del 619 bp	CAA TGT ATC ATG CCT CTT TGC ACC	2	242
Cd 15 (G-A)	TGA GGA GAA GTC TGC CGT TAC TGC CCA GTA	4	500
Cd 5 (-CT)	ACA GGG CAG TAA CGG CAG ACT TCT CCG CGA	3	205
Cd 30 (G-C)	TAA ACC TGT CTT GTA ACC TTG ATA CCT ACG	3	280
Cd 30 (G-A)	TAA ACC TGT CTT GTA ACC TTG ATA CCT ACT	3	280
Fr 16 (-C)	TCA CCA CCA ACT TCA TCC ACG TTC ACG TTC	3	238
IVSII-1 (G-A)	AAG AAA ACA TCA AGG GTC CCA TAG ACT GAT	3	634
Cd 26 (G-T)(Hb-E)	TAA CCT TGA TAC CAA CCT GCC CAG GGC GTT	3	278
Cap +1 (A-C)	ATA AGT CAG GGC AGA GCC ATC TAT TGG TTC	4	567
Fr 47-48 (+ATCT)	ATA ACA GCA TCA GGA GTG GAC AGA TAG ATC	3	467
1VS1-25 (25b del)	CTC TGG GTC CAA GGG TAG ACC ACC AGC ATA	3	354
Hb-S	CCC ACA GGG CAG TAA CGG CAG ACT TCT GCA	3	207
1. CONTROL	CAA TGT ATC ATG CCT CTT TGC ACC		
2. CONTROL	GAG TCA AGG CTG AGA GAT GCA GGA		
3. COMMON	ACC TCA CCC TGT GGA GCC AC		
4. COMMON	CCC CTT CCT ATG ACA TGA ACT TAA		

Table: 3.3. Primers used for identifying the normal alleles of the respective mutations.

Allele:	Primer for the normal sequence:	Used with:	Frag Size:
IVSI-5	CTC CTT AAA CCT GTC TTG TAA CCT TGT TAC	3	285
Fr 8-9	CCT TGC CCC ACA GGG CAG TAA CGG CAC ACT	3	214
IVSI-1	GAT GAA GTT GGT GGT GAG GCC CTG GGT AGG	4	450
Fr 41-42	GAG TGG ACA GAT CCC CAA AGG ACT CAA AGA	3	443
Cd 15	TGA GGA GAA GTC TGC CGT TAC TGC CCA GTG	4	500
Cd 5	ACA GGG CAG TAA CGG CAG ACT TCT CCG CAG	3	207
Cd 30	TAA ACC TGT CTT GTA ACC TTG ATA CCT ACC	3	280
IVSII-1	AAG AAA ACA TCA AGG GTC CCA TAG ACT GAC	3	634
Fr 16	TCA CCA CCA ACT TCA TCC ACG TTC ACG TTG	3	238
1. CONTROL	CAA TGT ATC ATG CCT CTT TGC ACC		
2. CONTROL	GAG TCA AGG CTG AGA GAT GCA GGA		
3. COMMON	ACC TCA CCC TGT GGA GCC AC		
4. COMMON	CCC CTT CCT ATG ACA TGA ACT TAA		

Electrophoresis of ARMS products:

At the end of PCR 20µl of the amplified product was mixed with 5µl of the loading dye (0.25% bromophenol blue in 40% sucrose). Electrophoresis was carried out in 4% NuSieve Agarose gel (FMC Corporation, USA) at 150 V for 1 hour. The gels were stained in ethidium bromide (250µg/100ml) for 30 minutes and were visualized and photographed under 302 nm UV light.

Multiplex ARMS PCR:

A multiplex PCR for ARMS (Newton and Graham 1994) was carried out under the standard PCR conditions but instead of using one ARMS primer specific for one mutation, several primers were mixed. The combination of ARMS primers used for screening in multiplex PCR is presented in Table: 3.4. The three multiplex primers, AD-1, AD-2 and AD-3, were prepared as stock solutions containing 5pmol/µl each of all the primers for the mutant alleles. In addition, the two control primers and the respective common primer were also added to the stock mixture. In each ARMS reaction 1µl of the stock primers was used.

The amplified products of all mutations were sufficiently different for resolution by polyacrylamide gel electrophoresis. However, the size of the fragments generated by IVSI-1 and IVSI-5 differed by 5 bp and therefore was difficult to resolve. Similarly there was no difference between the fragments of Cd30 G-C and G-A and IVSI-1. The problem of differentiating IVSI-1 and IVSI-5 was overcome by adding IVSI-1 primer to AD-1 and AD-2 groups. IVSI-5 resulted in amplification with AD-1, but IVSI-1 caused amplification with AD-1 and AD-2. Amplification with AD-2 but not AD-1 indicated Cd30. The difference between the two mutations in Cd30 was only of academic interest because the same normal primer was required to differentiate between the homozygotes and heterozygotes. AD-3 primer combination included Cd15 and Cap+1 as their common primer was different from the one used with other mutations (Table 3.2).

An allelic ladder for the respective mutations was prepared by pooling together the PCR products of separately amplified reactions for various mutations. Each fragment was amplified in 50µl reaction mixture under standard ARMS conditions. The individually

amplified products were pooled and were kept frozen in aliquots. 5µl of the pooled product was used in all polyacrylamide gel electrophoresis runs.

Table: 3.4. Combination of various mutations screened by multiplex ARMS PCR.

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

<u>*Electrophoresis of multiplex ARMS products:*</u>

The multiplex ARMS amplified products were resolved by non-denaturing polyacrylamide gel electrophoresis on Mini-Protean electrophoresis apparatus (Bio-rad, USA). 2 µl of the amplified product was loaded on 6% non-denaturing polyacrylamide gels measuring 1mm X 10cm X 10cm. Electrophoresis was carried out at 150V for 40 minutes. The gels were stained by silver nitrate. The details of gel preparation and silver staining are described in the subsequent sections.

Denaturing Gradient Gel Electrophoresis (DGGE):

β-globin gene was amplified in several overlapping fragments as shown in Fig: 3.1. Table: 3.5 shows the sequence of the primers used for amplification of various fragments of the β-gene. The primers were in use at the Perinatal Centre, UCH, London.

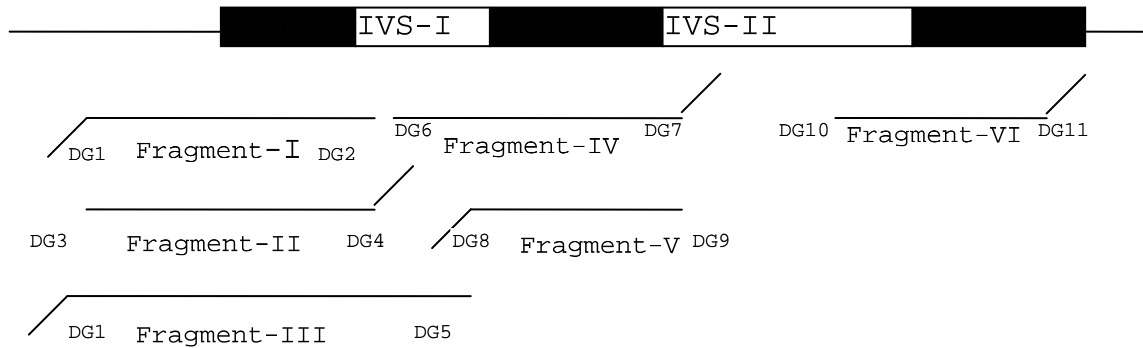

Fig: 3.1. Fragments of β-globin gene that were amplified for DGGE.

PCR conditions for DGGE:

PCR for DGGE was carried out in a 50μl reaction mixture containing 10 pM of each primer, 1.0 unit of Taq polymerase (Perkin Elmer, UK), 200 μM of each dNTP (Boehringer Manheim), 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 mg/ml gelatin and 0.3-0.5 μg of genomic DNA. Thermal cycling consisted of initial denaturation for 5 minutes at 95°C and then 40 cycles each of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, and DNA extension at 70°C for 1 minute. In the final cycle the extension was prolonged to 3 minutes. 10μl of the amplified product was run on 2% agarose gel to determine the quality of amplification.

Preparation of denaturing gels:

The following stock solutions were prepared:

1. 40% acrylamide (Molecular Biology grade. BDH, UK) and 1.07% bisacrylamide (Sigma Chemicals, UK).
2. 20 X gel running buffer (TAE pH 7.4) containing 800 mM Tris base, 400 mM Sodium acetate, and 20 mM EDTA (BDH, UK).
3. 80% denaturant stock solution containing 7% acrylamide, 32% formamide (BDH, UK), 5.6 M Urea (BDH, UK) and 1 X TAE buffer (pH 7.4).
4. 0% denaturant stock solution containing 7% acrylamide, and 1 X TAE buffer (pH 7.4).

Formamide was deionized before use by gently stirring with Dowex AG50W (20-40) mesh Mixed bed resin (Bio-rad, UK). The stock solutions of 0% and 80% denaturent were used to

prepare the gradients of varying strengths. Gels measuring 20 X 16 cm and 1.5 mm in thickness were poured by using a gradient mixer (Hoefer Scientific, USA). Ammonium persulphate (100µl of 10% stock solution) and 10µl of TEMED (Sigma Chemical, UK) were used as gel polymerization catalysts.

Formamide urea gradient varied for different fragments of the β-globin gene. The optimal gradient was determined by trial and error. The fragments-I, II and III were run on a denaturant gradient of 42% and 72%. The fragment-IV and V were run on 50%-80% gradient and the fragment-VI was run on 25%-50% gradient.

Table: 3.5. DGGE primers for amplification of various fragments of β-globin gene.

Frag-ment:	ID	Location:	Sequence:	Size:
I:	DG1	-123 to -104	5' 40[GC] CTG TCA TCA CTT AGA CCT CA	286 bp
	DG2	Exon-I Cd 24-17	5' CAA CTT CAT CCA CGT TCA CC	
II:	DG3	-129 to -104	5' GTA CGG CTG TCA TCA CTT AGA CCT CA	297 bp
	DG4	Exon-I Cd 24-17	5' 45[GC] CAA CTT CAT CCA CGT TCA CC	
III:	DG1	-123 to -104	5' 40[GC] CTG TCA TCA CTT AGA CCT CA	424 bp
	DG5	IVSI-119 to 101	5' AAA ATA GAC CAA TAG GCA G	
IV:	DG6	IVSI-29 to 48	5' AAG GAG ACC AAT AGA AAC TG	409 bp
	DG7	IVSII-30 to 11	5' 45[GC] AGA AAA CAT CAA GGG TCC CA	
V:	DG8	IVSI-101 to 120	5' 40[GC] CTG CCT ATT GGT CTA TTT TC	321 bp
	DG9	IVSII-30 to 11	5' AGA AAA CAT CAA GGG TCC CA	
VI:	DG10	IVSII 588 to 607	5' ATG ATA CAA TGT ATC ATG CC	339 bp
	DG11	Exon-III Cd 132-125	5' 45[GC] TCT GAT AGG CAG CCT GCA CT	

GC Clamp
45[GC] GCGGGCGGGGCGGGGGCACGGGGGGCGCGGCGGGCGGGGCGGGGG
40[GC] GCGGGCGGGGCGGGGGCACGGGGGGCGCGGCGGGCGGGGC

<u>*Running conditions for DGGE:*</u>

Electrophoresis was carried out on SE600 vertical polyacrylamide gel running system (Hoefer Scientific, USA). The original buffer bath was replaced by a specially designed acrylic container designed to hold the gel assembly as well as the heating and the buffer circulation device. Once the gradient gel had polymerized, the comb was carefully removed and the wells were washed with 1 X gel running buffer. Each well was loaded with 7µl of amplified product mixed with 6µl of loading dye. The quantity of amplified product was

increased to 8-10μl depending on the quality of amplification. Once loaded, the gel was removed from the casting assembly and the upper buffer reservoir of the system was secured in place. The Hoefer SE600 accommodates two gels at a time. The whole assembly was placed in the main buffer compartment and the heating and buffer circulating device was turned on.

Electrophoresis was carried out at 50V for 16 hours. Throughout the procedure buffer temperature was maintained at 60°C. The gels were then removed and the glass plates carefully separated. The gel was stained in ethidium bromide (250μg/100 ml) solution for 30 minutes. It was photographed at 302 nm UV light.

Genomic sequencing:

The primers used for amplifying the β-globin gene and the internal sequencing primers are shown in Table: 3.6.

PCR amplification for sequencing:

The whole of β-globin gene (-308 bp from the Cap site to +475 bp from the termination site) was amplified by a set of primers in which the 5' primer was biotinylated (Table: 3.6). PCR was carried out in 100 μl reaction mixture containing 20 pM each of the two primers and 2 units of Taq polymerase (Perkin Elmer, UK). The thermal cycling consisted of 1st cycle of denaturation at 94°C for 4 minutes, primer annealing at 56°C for 3 minutes and extension at 72°C for 2 minutes. In the subsequent 35 cycles the timing of denaturation and primer annealing were reduced to 1 minute and 2 minutes respectively. In the last cycle the extension reaction was prolonged to 8 minutes. The quality of amplification was checked by running 10μl of the product on 2% agarose gel.

Preperation of single stranded template DNA:

DNA amplified with biotinylated primer was used for preparation of single-stranded template. Streptavidin coated Dynabeads (M-280, Dynal UK) and a magnetic particle concentrator (MPC-E, Dynal UK) was used to separate and concentrate the single stranded DNA (Hultman et al, 1989; Thein and Hinton 1991). The dynabeads were washed in TES (10 mM Tris-HCl, 1 mM EDTA, and 100 mM NaCl at pH 8.0). Approximately 80μl of the

amplified product was mixed (without vortexing) with 50µl of dynabeads. It was left on the bench for 5 minutes followed by denaturation with freshly prepared 0.15 M NaOH. The denatured single stranded template was washed in TES and concentrated to yield a final volume of 20µl.

Preparation of double stranded template DNA:

For the majority of the sequencing reactions double stranded DNA template was used. The amplified double stranded DNA contains excess of unused amplification primers, dNTPS and some extraneous single stranded DNA produced by PCR. Two hydrolytic enzymes i.e. Shrimp alkaline phosphatase, and Exonuclease-I can remove the unwanted accompaniments. The two enzymes are available in a kit (United States Biochemical, USA). In the protocol, 5µl of the amplified product was treated with 2 units of Shrimp alkaline phosphatase and 10 units of Exonuclease-I. The reaction mixture was incubated at 37°C for 15 minutes in a thermal cycler followed by an additional 15 minutes at 80°C to inactivate of the two enzymes.

Labelling reaction:

The labelling reaction was done by the Sequenase version 2.0 sequencing kit (United States Biochemical, USA). The method described by Tabor and Richardson (1990), based on di-deoxy chain termination method was used. The enzyme treated PCR product was mixed with 5 pM of internal sequencing primer (Table: 3.6) and water to yield a final volume of 10µl. It was denatured at 100°C in a thermal cycler for 2-3 minutes. It was then cooled as quickly as possible by directly submerging the reaction vial in crushed ice for 5 minutes.

The following were added to the ice cold denatured DNA: 1µl of 0.1 M dithiothreitol (DTT), 2µl of 1:5 diluted labelling mix, 0.5µl of ^{35}S dATP (5 mCi) (Amersham UK), 2µl reaction buffer, and 2µl of diluted Sequenase DNA polymerase. The reaction mixture was incubated at room temperature for 5 minutes. In the mean while four tubes containing 2.5 µl termination analogues (G, A, T and C) were warmed at 37°C for 1 minute. 3.5µl of the labelling reaction was added to the side of each of the four termination analogue tubes avoiding direct contact between the two. The reaction tubes were quickly centrifuged for a few seconds to allow mixing of the labelling reaction and the termination analogues. The

tubes were incubated for 5-10 minutes at 37°C. The reaction was stopped by adding 4µl of stop solution (95% formamide, 0.05% Xylene Cyanol and 0.05% Bromophenol blue). The reaction tubes were frozen until electrophoresis.

Table: 3.6. The primers and their sequence used in sequencing the β-globin gene.

Primers used for amplification of β-globin gene:

5'(Biotinylated) -307 from Cap site:	5′ CGA TCT TCA ATA TGC TTA CCA AG
3' +475 from termination site:	5′ GAG TCA AGG CTG AGA GAT GCA GGA

Internal sequencing primers:

ID:	**Location:**	**Sequence:**
SE1	Cd 16	5' CCC ACA GGG CAG TAA CGG CAG ACT TCT CCT
SE2	Cd 33	5' ACC AGC AGC CTA AGG GTG GGA AAA TAG ACC
SE3	IVSII-24	5' ACA TCA AGG GTC CCA TAG AC
SE4	IVSII-142	5' GAT CCT GAG ACT TCC ACA CT
SE5	IVSII-332	5' ATG TAC TAG GCA GAC TGT GT
SE6	IVSII-742	5' GGA TTG TAG CTG CTA TTA GC
SE7	Cd 142	5' CCA GGG CAT TAG CCA CAC CA
SE8	+196 from term site	5' AAT GCA CTG ACC TCC CAC ATT CC

Preparation of sequencing gels:

The sequencing gels were prepared at 6% polyacrylamide concentration (Acrylamide and Bis-acrylamide ratio 19:1). Both the reagents used were of molecular biology grade (BDH and Sigma Chemicals, UK). Molecular biology grade urea (BDH, UK) at 7M concentration was used as a denaturant. The glass plates were thoroughly washed with light detergent and de-ionized water. The plates were dried and cleaned with ethanol. One of the plates, usually the same plate for all gels, was siliconized with Sigmacoat (Sigma Chemicals, UK). The gel thickness was kept at 0.4 mm. All sequencing gels were run on an apparatus from Bio-rad, USA. This can accommodate gels measuring 20cm X 50cm. Ammonium persulphate (400µl) and TEMED (40µl) were used as polymerization catalysts. The gels were poured using a 50ml syringe and keeping the plates inclined at an angle of about 45°. Any bubbles were removed by gently tapping the plates. Shark-tooth combs were used for creating the sample wells.

Running the sequencing gels:

The gels were run in TBE buffer (Tris 10.8g, Borate 5.5g and Na_2EDTA 0.93g at pH 8.3). The sample wells were thoroughly washed with gel running buffer to remove any urea that constantly flows out of the gel. The labelled samples were denatured at 95°C for 2-3 minutes and kept on ice until loading. Each well was loaded with 2.5-3.0μl of reaction mixture from each tube and electrophoresis was carried out at a constant power of 50W for 2-3 hours. During the run the gel temperature remained at 50°C. At the end of the run the plates were carefully separated and the gel was fixed for 20 minutes with freshly prepared fixative containing 10% methanol and 10% acetic acid. The fixed gel was transferred to a piece of Whatman filter paper cut to a size slightly larger than the glass plates. The gel was covered with cling film and placed on a gel dryer at 80°C for 1 hour. The gel was then exposed to X-ray film (Kodak, USA) for 2-3 days.

Restriction Fragment Length Polymorphism (RFLP) analysis:

The polymorphic sites linked to the β-globin gene were analysed by first amplifying the fragment of DNA containing the site, followed by its digestion with a restriction enzyme. Table: 3.7 gives details of the polymorphic sites, the primer used for amplification and the restriction enzyme used. All of the primers were in use at the Perinatal centre, UCL, London. The general conditions for PCR were the same as ARMS except that the annealing temperatures of the primers for Aγ, 5'ψβ, 3'ψβ, Rsa-I β and Hinf-I β was 56°C and 60°C for Gγ. The number of cycles was also increased to 30. Cleavage was carried out by overnight incubation of 20μl of the amplified product at 37°C with 10 units of the restriction enzyme (Gibco BRL, UK). The digested fragments were separated on 4% NuSieve Agarose gel.

Xmn-I Polymorphism:

Xmn-I can recognize the C-T polymorphism at position -158 from the cap site of the Gγ-globin gene (Thein et al, 1988). In order to demonstrate this polymorphism, a 641bp fragment of DNA flanking the polymorphism was amplified using the following primers:

5’-GAA CTT AAG AGA TAA TGG CCT AA
5’-ATG ACC CAT GGC GTC TGG ACT AG

PCR conditions were the same as for the RFLP protocol except that the annealing temperature was increased to 60°C. The amplified fragment was digested with 10 units of Xmn-I (Boehringer, Mannheim) and the results were read after electrophoresis on 4% Nusieve Agarose gel.

Table: 3.7. Polymorphic sites linked to the β-globin gene, the restriction enzymes used for their recognition and the sequence of the primers used for amplification of the respective fragments.

Site:	Enzyme used:	Primer sequence:
Gγ-gene	Hind-III	5'-AGT GCT GCA AGA ACA ACT ACC 5'-CTC TGC ATC ATG GGC AGT GAG CTC
Aγ-gene	Hind-III	5'-GAC TAG TGC TTG AAG GGG AAC AAC 5'-CCT CTG CTG ATT CAT TTC TTA CAC
5'ψβ-gene	Hind-II	5'-TCC TAT CCA TTA CTG TTC CTT GAA 5'-ATT GTC TTA TTC TAG AGA CGA TTT
3'ψβ-gene	Hind-II	5'-GTA CTC ATA CTT TAA GTC CTA ACT 5'-TAA GCA AGA TTA TTT CTG GTC TCT
β-gene	Hinf-I	5'-TGG ATT CTG CCT AAT AAA A 5'-GGG CCT ATG ATA GGG TAA T
β-gene	Rsa-I	5'-AGA CAT AAT TTA TTA GCA TGC ATG 5'-ACA TCA AGG GTC CCA TAG AC

Gene analysis for α-thalassaemia:

Most α-thalassaemias are caused by large gene deletions. In the past these deletions were detected by laborious and expensive restriction enzyme mapping of the gene. However, the introduction of PCR has simplified the detection of gene defects in α-thalassaemia. The common α-thalassaemia-1 and 2 determinants can now be detected by PCR (Bowden et al, 1992; Dode et al, 1992; Baysal and Huisman 1994). The PCR primers are used to amplify appropriate segments of the chromosome with the deletion and the normal chromosome under identical experimental conditions. On a normal chromosome the PCR primers are far apart and therefore are unable to amplify whereas the presence of an α-thalassaemia deletion brings the two primers sufficiently close for the amplification to take place. The amplified products can be separated by agarose gel electrophoresis.

PCR conditions for -$\alpha^{3.7}$ and -$\alpha^{4.2}$ detection:

The PCR buffer contained 67 mM Tris-HCl, pH 8.8; 16.6 mM ammonium sulphate, 0.10 mg/ml Bovine Serum Albumin, 10 mM β-Mercaptoethanol, 4.0 mM $MgCl_2$, 10% DMSO, 200 μM dNTPs, and 2.0 units of Taq polymerase (Perkin Elmer, UK). The primers and their sequences are presented in Table: 3.8. The strategy for detection is described in Fig: 3.2 (Baysal and Huisman 1994). Normally the primers A+B are far apart and can not amplify. Whereas the presence of -$\alpha^{3.7}$ deletion brings the two primers sufficiently close for amplification to take place. In the absence of the -$\alpha^{3.7}$ deletion primers A+C amplify the normal sequence. A similar approach was used for -$\alpha^{4.2}$, where primers D+E amplify the abnormal fragment if present, and D+F amplify the normal sequence.

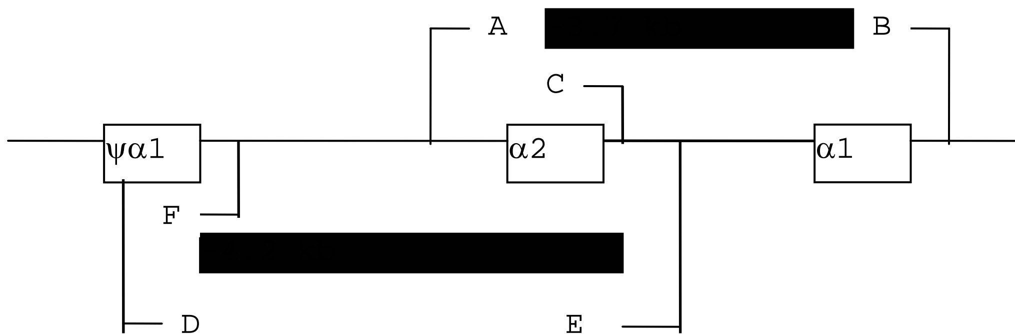

Fig: 3.2. Strategy for the amplification of segments of DNA from chromosomes with the two types of α-thalassaemia-2 deletions. The functional α2 and α1 and ψα1 genes are shown as open boxes. The solid boxes indicate the extent of the deletions. Primers A and C amplified a control fragment whereas primers A and B amplified only when 3.7kb deletion was present. Similarly, primers D and F amplified a control fragment while primers D and E amplified a fragment only when 4.2kb deletion was present (Baysal and Huisman 1994).

The PCR for detection of normal and mutant allele for -$\alpha^{3.7}$ was carried out in two different tubes because the amplified product under both conditions is of the same size, and therefore, cannot be separated on agarose gel electrophoresis. For the -$\alpha^{4.2}$ both the reactions were done in the same tube, as the amplified products considerably differ in size. The PCR was carried out with the “Hot start” method where Taq polymerase was added when reaction mixture was above 80°C. The thermal cycling consisted of 25 cycles each of denaturation at 94°C for 1 minute, primer annealing at 60°C for 1 minute and extension

reaction at 72°C for 3 minutes. The extension in the last cycle was prolonged to 8 minutes. At the end of the PCR the amplified products were separated on 2% agarose gel.

Table: 3.8. Primers used for detection of α thalassaemia deletions (Baysal and Huisman 1994).

Primer ID:	**Sequence:**	**Concentration:**
A:	CTT TCC CTA CCC AGA GCC AGG TT	25 pmol/reaction
B:	CCC ATG CTG GCA CGT TTC TGA GG	25 pmol/reaction
C:	CCA TTG TTG GCA CAT TCC GGG ACA	25 pmol/reaction
D:	CCT TCC TCT CAC TTG GCC CTG AG	20 pmol/reaction
E:	CCC TGG GTG TCC AGG AGC AAG CC	15 pmol/reaction
F:	CGC CTC CCT GGA CAA GTT	15 pmol/reaction

Analysis of Short Tandem Repeats (STR):

A bulk of intergenic DNA in the human genome consists of tandemly repeating sequences whose length varies between individuals in the same population. The polymorphisms created by such elements are termed variable number of tandem repeats (VNTR) for the larger repeats and short tandem repeats (STR) for 1-6 base pair repeats (Krawczak and Schmidtke 1994). VNTRs and STRs can be analyzed by PCR (Horn et al, 1989; Urquhart et al, 1994).

STR alleles at D21S11 locus were amplified by the following pair of flanking primers (Sharma and Lit 1992):

D21S11-F 5'-GTG AGT CAA TTC CCC AAG-3'

D21S11-R 5'-GTT GTA TTA GTC AAT GTT CTC C-3'

The PCR was carried out in a 25µl reaction mixture under the same conditions as for the ARMS except that a hot start strategy was used. The thermal cycling comprised of 30 cycles each of denaturation at 93°C for 48 seconds, primer annealing at 60°C for 48 seconds and extension reaction at 72°C for 1 minute.

Allele sizing of D21S11:

In order to carry out precise allele sizing for the D21S11 locus 30 samples were run on an automated gene scanning system called ALF (Pharmacia Biotech, USA). The DNA amplified with a fluorescent labelled primer is electrophoresed on a denaturing sequencing gel. An internal lane standard is also loaded along with the test sample. The internal lane standard as well as the amplified DNA is read by the flourescent detection system comprising of a laser source. It generates a signal that is recorded as a peak and is stored in a computer.

The samples analyzed on ALF were amplified with primers in which the forward primer was labelled with flourescene. The samples were loaded on a 6% polyacrylamide gel containing 8M urea. Two internal lane standards of 100 and 300 bp (Pharmacia Biotech, UK) were also loaded with each sample. The gel was run at 1900 Volts at 42°C until the equipment had recorded the samples and the lane standards.

Once the allele sizes of several samples had been accurately determined on the ALF, suitable samples that represented most of the common alleles were chosen as controls for manual allele sizing. Each amplified sample was run on two different types of polyacrylamide gels. The samples were first run on 6% denaturing polyacrylamide gels measuring 16 X 20 cm X 1.0 mm on a Protean-II apparatus (Biorad, UK). 7µl of the amplified product and 5µl of loading dye was loaded and overnight electrophoresis was carried out at 100 volts. The gels were stained in ethidium bromide for 30 minutes and were photographed under 302 nm UV light.

The samples were also run on 6% denaturing polyacrylamide sequencing gels. The protocol for preparing the gels was the same as for sequencing, except that larger sample wells were created by using widely spaced combs. This was essential to accommodate 5µl of the sample and the loading dye. The gels were run at 500 Volts overnight.

Silver staining of polyacrylamide gels:

The glass plates were carefully separated, and the gel that usually sticks to one of the plates was fixed in 30-50 ml of freshly prepared fixing solution, containing 10% ethanol and 5% glacial acetic acid. Wrinkles in the gel, if any, were removed by gently blowing the fixative

on the wrinkles with the help of a pasture pipette. The gel was then evenly covered with 0.1% silver nitrate for 20 minutes. The stain was poured off and the reaction developed by adding freshly prepared solution of 1.5% NaOH and 0.15% formaldehyde. Within 10-15 minutes the bands of DNA could be seen clearly. The gel was then washed with tap water and transferred to a clean sheet of filter paper. It was covered by cling film and left between layers of filter paper to dry.

Variable Number of Tandem Repeat (VNTR) analysis:

The VNTR sequences in the Apolipoprotein-B gene were amplified by PCR (Deka et al, 1992). The general conditions of PCR were the same as for ARMS PCR except that the annealing temperature was reduced to 55°C and the number of cycles was increased to 30. The following primers were used to amplify the VNTR sequences (Deka et al, 1992):

ApoB-F: 5'-ATG GAA ACG GAG AAA TTA TG

ApoB-R: 5'-CCT TCT CAC TTG GCA AAT AC

Electrophoresis of the amplified fragments was carried out on 2% agarose gels at 40V overnight. The gels were stained in ethidium bromide and were photographed at 302nm.

Statistical analysis:

Means, ranges, and standard deviations were calculated for the continuous variables that had normal distribution. Confidence intervals and hypothesis testing for the data that were normally distributed and for the data that were not normally distributed were calculated by parametric and non-parametric tests respectively (Altman 1991). A computer package "SPSS version 6.1.3" was used for most of the statistical analysis.