**Title: Mapping the Mutation Spectrum in Prenatal Thalassemia: Insights from a Tertiary Care Center in Delhi**

**Authors: Komal Uppal, Jyotsna Gaur, Sunil Kumar Polipalli, Somesh Kumar, Sangeeta Gupta, Seema Kapoor**

**MATERIALS AND METHODS**

**ARMS PCR:**

Heterozygous beta-thalassemia detected by HPLC method were subjected to molecular DNA analysis. The first screening for common beta-thalassemia mutations was performed using the ARMS PCR technique. Samples that tested negative for these mutations underwent DNA Sanger sequencing to identify the causative beta -thalassemia mutation. Genomic DNA was extracted by using Qiagen blood The sequence of primers for the analysis was presented in the internal control and general primer table.

PCR was performed in a thermal cycler veriti, Applied Biosystems. Q5® Hot Start High-Fidelity 2X Master Mix was used. The PCR procedure was performed as per the given protocol. Two vials were taken for mutation detection, one tube for normal N and one for mutant M. 12.5µl Q5® Hot Start Master mix was added to 1.5 ml microcentrifuge vials. 5.0 µl of molecular grade water was added to both N and M tubes. 1.0 µl internal control primer 1, internal control primer 2 were added to normal and mutant tubes 1.0 µl primer A or B were added to normal and mutant vials as needed, depending on the mutation to normal. 1.0 µl of mutant primer was added to the mutant tube. 5.0 µl of genomic DNA was added to each tube. PCR amplification was performed with the following conditions Denaturation at 94°C for 1min followed by 1 cycle of annealing at 63°C for 1.0 min with a final extension at 72°C for 3 min. After PCR, the PCR products were subjected to electrophoresis in 1.5% agarose gel at 100V for 45 min. The gel is visualized under UV light. Interpretation was made based on the observed mutation -specific band: samples can be identified as normal, heterozygous or homozygous.

**Sanger sequencing:** Direct Sanger sequencing is the method of choice for detecting rare and novel mutations. The sequencing method was performed with the help of 3500 Genetic Analyser. The human beta- globin gene was sequenced to identify causative mutations which were not detected by the ARMS PCR protocol. DNA sequence analysis of the HBB region was performed with Three sets of overlapping primers which were designed to amplify the complete DNA sequence. Thus, three PCR were performed for each sample. Primer design was performed online by using primer 3 software. Three sets of overlapping primers covering the entire HBB region was prepared (Annexure-I).

The HBB gene region was amplified using PCR, with three tubes processed for each sample. Three primers were used with Q5®HotStart High-Fidelity 2X Master mix, template DNA, and adjusted volume. The PCR mix was added to a plate and cycled in a thermal cycler. A distinct 600 bp PCR amplicon band was observed on gel. Enzymatic purification of amplified product with the help of ExoSap-IT Reagent, sequenced using forward or reverse primers, and analysed for mutations using NCBI Blast online tool. Short nucleotide variations and Single nucleotide polymorphism were identified using sequence BLAST. Variants were identified by Human Genome Variation Society. SNP accession numbers were indicated for DNA sequence variations. Data were analysed statistically using SPSS version 11.5. Overall, the process involved amplification, sequencing, mutation identification, and statistical analysis of DNA samples from the target region.

**CONTROL AND COMMON PRIMERS**

1. COMMON A 5’ ACC TCA CCC TGT GGA GCC AC
2. COMMON B 5’ CCC CTT CCT ATG ACA TGA ACT TAA
3. CONTROL FORWARD 5’ GAG TCA AGG CTG AGA GAT GCA GGA
4. CONTROL REVERSE 5’ CAA TGT ATC ATG CCT CTT TGC ACC

 **Beta Thalassemia primers**

|  |  |  |  |
| --- | --- | --- | --- |
| Sequence Name | Sequence 5' – 3' |  | Fragment Size /bp |
| IVS 1-5 (G-C) | N - CTC CTT AAA CCT GTC TTG TAA CCT TGT TAC |  | 285 |
|  | M- CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG |  | 285 |
| IVS 1-1 (G-T) | N- GAT GAA GTT GGT GGT GAG GCC CTG GGT AGG |  | 454 |
|  | M- TTA AAC CTG TCT TGT AAC CTT GAT ACG AAA |  | 281 |
| CD 41/42 (-CTTT) | N- GAG TGG ACA GAT CCC CAA AGG ACT CAA AGA |  | 443 |
|  | M- GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT |  | 439 |
| CD 8/9 (+G) | N-CCT TGC CCC ACA GGG CAG TAA CGG CAC ACT |  | 214 |
|  | M-CCT TGC CCC ACA GGG CAG TAA CGG CAC ACC |  | 215 |
| CD 15 (G-A) | N-TGA GGA GAA GTC TGC CGT TAC TGC CCA GTG |  | 500 |
|  | M-TGA GGA GAA GTC TGC CGT TAC TGC CCA GTA |  | 500 |
| Cap site +1 (A-C) | N-ATA TGT CAG GGC AGA GCC ATC TAT TGG TTA |  | 582 |
|  | M-ATA TGT CAG GGC AGA GCC ATC TAT TGG TTC |  | 582 |
| CD 30 (G-C) | N- 5' TAA ACC TGT CTT GTA ACC TTG ATA CCT ACC |  | 280 |
|  | M- 5'TAA ACC TGT CTT GTA ACC TTG ATA CCT ACG |  | 280 |
| CD 30 (G-A) | N- 5' TAA ACC TGT CTT GTA ACC TTG ATA CCT ACC |  | 280 |
|  | M- 5'TAA ACC TGT CTT GTA ACC TTG ATA CCT ACT |  | 280 |
| IVS 1-1 (G-A) | N- TTA AAC CTG TCT TGT AAC CTT GAT ACC CAC |  | 281 |
|  | M- TTA AAC CTG TCT TGT AAC CTT GAT ACC GAT |  | 281 |
| CD 5 (-CT) | N- ACA GGG CAG TAA CGG CAG ACT TCT CCG CAG |  | 204 |
|  | M- ACA GGG CAG TAA CGG CAG ACT TCT CCG CGA |  | 202 |

**Sanger Sequencing Primers for HBB gene**

First amplicon:

HBB For1 primer TGGAGCCACACCCTAGGGTT Tm – 62.5°C

HBB Rev1 primer TGCAATCATTCGTCTGTTTCCCAT Tm - 62°C

 Second amplicon:

HBB For2 primer GAGTCTATGGACGCTTGATG Tm – 61.2°C

HBB Rev2 primer GCTATTGCCTTAACCCAGAAATT Tm – 61.2°C

Third amplicon:

HBB For3 primer ACTTTCCCTAATCTCTTTCTTTAG Tm – 60.9°C

HBB Rev3 primer TTATGTTTAAATGCACTGACCTCC Tm – 60.9°C