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Whole Gene Sequencing Based Screening Approach to Detect β-Thalassemia Mutations

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Abstract

About 200 causative mutations are characterized in the *β-globin* gene. Beta thalassemia diagnosis is very complicated due to the genetic diversity of HBB gene across different geographical regions of the world. In the present study, we have analyzed 138 clinical specimens among them 66 were from 21 unrelated families (trio samples which had DNA from father, mother and chorionic villus sample/amniotic fluid sample) and 72 individual specimens using newly developed sequencing and PCR based assay. We observed 11 different HBB gene mutations in 138 samples, which were also cited by literature as the most prevalent mutations in Indian sub-continent population. The most common mutation observed in our study was HBB.C.92+5 G>C (GC+CC genotype was observed to be 44.93%). Few interesting case studies like co-inheritance of sickle cell anemia and β-thalassemia traits, compound heterozygosity of beta thalassemia major mutation in the case of twin pregnancy were also focused briefly. Commercially available molecular diagnostic kits of HBB gene can detect and identify targeted mutations but will not detect novel and non-targeted mutations of beta thalassemia in parental blood and fetal samples. Hence, a screening technique involving complete sequencing of *HBB* gene (*β-globin* gene) is required along with gap PCR approach to provide complete diagnosis of beta thalassemia disease.

Keywords: Beta thalassemia; Sequencing; Chorionic villus sampling (CVS); Amniotic fluid (AF); Prenatal screening

Introduction

Hemoglobinopathies are inherited genetic disorders of hemoglobin which includes beta-thalassemia (β-thalassemia), alpha thalassemia, sickle cell anemia etc. Around 4.5% of world population is affected by these disorders whereas in India 25 million population is carrier for the same [1,2]. Although, Indian population is very well diversified with more than 3000 ethnic groups, genetic diseases are a big threat due to social beliefs, rituals and traditions of marriage [3]. Thalassemia is the most common human genetic disorders with more than 270 million carriers and 350,000 thalassemia major patients worldwide [4]. The prevalence of thalassemia in India is 3-8%, including 10,000 babies with β-thalassemia major being born every year which comprises of 10% of the total number in the world [5,6]. As per the data published earlier, beta thalassemia was found specifically in high frequency in few communities like the Sindhis, Kutchhis, Bhanushalis and Punjabis from Western and Northern India (5-15%) [7-9] whereas north eastern regions have high prevalence of HbE (5–50%) [10-12].

HBB gene contains three coding exons separated by two introns with the size of around 1600bp which has been conserved throughout the evolution process. β-thalassemia is caused by 21 important mutations out of 200 point mutations and indels published worldwide [13,14]. These mutations are present both within the *β-globin* gene (*HBB*) and also in regulatory elements. During splicing mechanism, abnormal mRNA splicing also occurs which results in nonfunctional mRNA. Therefore, it is imperative to sequence full *HBB* gene to identify novel mutations or variants which have not been included in the available commercial kits. The proposed study is focused to develop a screening assay for identification of mutations associated to β-thalassemia using nucleotide sequencing technology.

Materials and Methods

Details of clinical specimens

In present study, we collected 138 clinical specimens, among them 66 samples were from 21 unrelated families and 72 individual samples. In total, there were 61 males, 54 females and 23 fetal samples for whole gene sequencing assay. Blood samples of all adults were received in EDTA vaccutainer and Chorionic Villus Sample (CVS)/Amniotic fluid (AF) of fetal samples were obtained with consent of parents for beta thalassemia screening. Among 66 samples, 21 families were from Western India and two families were from Bangladesh. All the individual samples were from western and northern Indian population. All clinical samples were drawn by clinicians and Gynecologists who are legally authorized to do so and obtained the consent forms. Personal details of samples, such as age, RBC indices and ethnicity were collected. We reconfirmed with the clinician about the availability of patient consent form in the hospital or clinic. Also, it was made clear to the clinician and parents that Xcelris Labs is abide by the Pre-Conception and Pre-Natal Diagnostic Techniques (PC & PNDT) Act of Government of India.

DNA extraction

Genomic DNA was extracted from 200 ul EDTA blood sample using standard QIAmp DNA Blood Mini-kit (Qiagen, Germany, Cat no. 51104) following the manufacturer's instructions. For CVS and Amniotic fluid samples, 4-5 ml of sample solution was centrifuged at 14000 rpm speed for 5 minutes to obtain pellet of cells which was processed using the same QIAmp DNA Blood Mini-kit protocol. Extracted genomic DNA was electrophoresed using 1% agarose gel. For quantification and purity check (A260/280) NanoDrop readings were taken on ND8000 Spectrophotometer model V 2.0.0 (Thermo Scientific Inc., USA). High quality DNA was extracted from all the 103 blood samples, 5 CVS and 11 amniotic fluid samples and DNA had absorbance ratio A260/280 between 1.8-2.0, which was further confirmed by agarose gel electrophoresis.

Primer design

Primers were designed with a modification of overhang extension at 5' end of forward and reverse oligonucleotide sequences to increase primer specificity [15]. Oligos were synthesized at Xcelris Labs Ltd., India. A total of six primers were chosen out of which two were forward primers P1 and P4, and four were reverse primers P2, P3, P5 and P6. These primers covered the whole *HBB* gene. Primer details are provided in Table 1 and pictorially represented in Figure 1.

S. No.	Primer	Primer sequence and orientation
1	P1 (forward)	5'- CTTAGAGGTTCATTGAATCACGGCTGTCATCACTTAGAC-3'
2	P2 (reverse)	5'-TATGACATATTTCGGATCGCCTCCCCTTCCTATGACATGA-3'
3	P3 (reverse)	5'-TATGACATATTTCGGATCGCAAGAGGTATGAACATGATTAGC-3'
4	P4 (forward)	5'- CTTAGAGGTTCATTGAATCGTGTACACATATTGACCAAATC-3'
5	P5 (reverse)	5'- TATGACATATTTCGGATCGCCAGATTCCGGGTCACTGTG-3'
6	P6 (reverse)	5'-TATGACATATTTCGGATCGCAATGCACTGACCTCCCACAT-3'

Table 1: Description of primers used for detection of β- thalassemia mutations in clinical specimen.

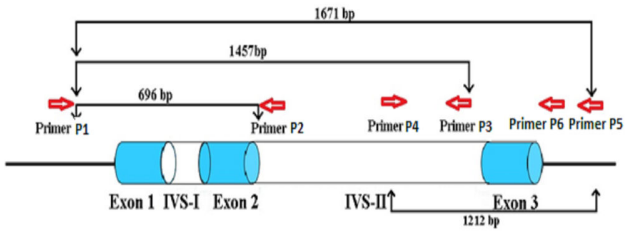

Figure 1: Schematic representation of HBB gene along with the details of primers and size of amplified PCR products (size of amplicons presented here is with respect to wild type samples).

Thermal cycling conditions for Primers P1-P2 and P4-P6

PCR amplification of β-globin gene was carried out using specific primer set P1(forward) and P2(reverse) using EmeraldAmp GT PCR Master Mix (Takara Clontech Labs Inc.) in 25 ul reaction volume. Thermocycling conditions consisted of 1 denaturing cycle at 96°C for 5 minutes followed by 35 cycles of denaturing at 94°C for 30 seconds, annealing at 62°C for 40 seconds, and extension at 72°C for 20 seconds. Final extension was at 72°C for 10 minutes using Applied Biosystems Veriti Thermal Cycler. Amplicons were electrophoresed on 2% agarose gel for quality check.

Nucleotide Sequencing using ABI3730xl

Amplified PCR products of set-1 primers P1(forward) - P2(reverse) were purified using ExoSAP-IT (USB, Cleveland, OH) before sequencing. Nucleotide Sequencing was done using a BigDye Terminator cycle sequencing kit and an ABI PRISM 3730xl DNA analyzer (Applied Biosystems, CA) [16]. Raw data obtained was analysed using bioinformatics software CLC Genomics.

Gap PCR for detecting Δ619bp Deletion using Primers P1-P3, P1-P5 and P4-P5

To detect Δ619bp deletion from β-globin gene, genomic DNA was subjected to Touch-Up Gradient PCR amplification using primer sets P1(forward) - P3(reverse), P1(forward) - P5(reverse) and P4(forward) - P5(reverse). Positive control, negative control and no template controls were used with every sample. Thermocycling conditions consisted of 1 denaturing cycle at 94°C for 4 minutes followed by 32 cycles of denaturing at 92°C for 45 seconds, annealing at 50°C for 7 seconds, 52.5°C for 7 seconds, 54°C for 7 seconds, 55.5°C for 7 seconds, 57°C for 8 seconds and 58.5°C for 8 seconds, extension at 72°C for 3.05 minutes. Final extension was at 72°C for 10 minutes (Applied Biosystems Veriti Thermal Cycler). PCR amplicons were checked on 2% agarose gel for gel based analysis (Figure 2).

Maternal contamination check

Several methods are available for determining the maternal contamination in the AF/CVS samples fluoroscently labeled micro satellite markers, radio labeled variable number of tandem repeats (VNTR) based markers, non-radio labeled VNTR markers etc. In present study we have used PCR based method for detecting the maternal contamination in the given sample using apoB VNTR primers by following the method described by Batanian et al. [16] for all the prenatal samples. In this method, AF or CVS sample was amplified along with parents sample and analyzed on 2% agarose gel electrophoresis. In which, maternal contamination can be identified by

presence of same allele in the AF/CVS sample as a banding pattern similar to maternal blood sample. Usually, one or two bands obtained upon amplification with apoB VNTR primer with each sample. For detecting contamination, we spiked the DNA of CVS/AF samples with 10% of maternal DNA and amplified them along with the non-spiked (pure DNA) and analyzed on agarose gel electrophoresis (Figure 3). All the trio samples were checked for MCC but here we have presented gel profile for one sample.

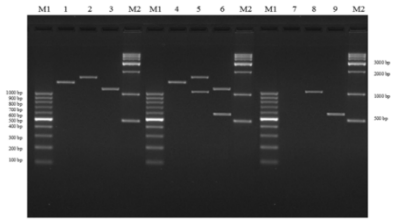

Figure 2: Gel-based identification of patients with Δ619 allele. Lane M1 and M2 denoted 100 bp and 1 kb ladder respectively. Lanes 1, 2 and 3 indicate a wild-type genotype on the basis of a 1457 bp amplicon from primer pair P1-P3, 1671 bp amplicon from primer pair P1-P5 and 1,212 bp amplicon from primer pair P4-P5 respectively. Lanes 4, 5 and 6 indicate a heterozygous genotype on the basis of a combination of the 1671 bp and 1052 bp amplicons from primer pair P1-P5 and the 1212 bp and 593 bp amplicons from primer pair P4-P5 respectively. Lanes 6, 7 and 8 indicate a homozygous mutant genotype on the basis of no amplicon from primer pair P1-P3 (as binding site for primer P3 falls in the deleted region of gene), 1052 bp amplicon from primer pair P1-P5 and 593 bp amplicon from primer pair P4-P5 due to deletion of 619 bp.

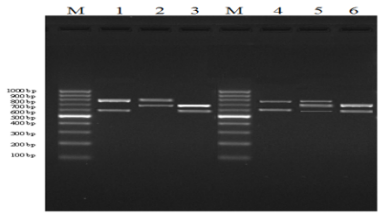

Figure 3: Gel based detection of maternal cell contamination (MCC) in the AF/CVS samples. Here, lane M indicates 100bp marker and lanes 1, 2 and 3 indicates paternal, maternal and AF of control sample and lanes 4, 5 and 6 denotes paternal, maternal and AF sample spiked with 10% maternal DNA.

Results

HBB gene mutation detection assay

The two phase assay was designed to cover all the mutations, insertions and deletions in the *HBB* gene. First phase, involved detection of mutations, insertions, deletions using sequencing technology and second phase involved gap PCR technology based identification of large deletions and insertions. *HBB* gene is made up of three exons separated by two intronic regions. Schematic presentation of *HBB* gene and the targeted region covered by the designed primers is given in the Figure 1. Sequencing method detected all the mutations as well as insertion/deletions up to 4bp, whereas large deletions such as Δ619bp deletion was detected using gap PCR.

In present study, primer pair P1-P2 generated 696bp of amplicon which covered all the mutations identified in this study. Primer sets P1-P3, P1-P5 and P4-P5 are designed to detect Δ619bp deletion. Sample with homozygous mutant allele for Δ619bp deletion, will generate the amplicon of 1052bp size with primer set P1-P5, a 593bp product with primers P4-P5 and will not amplify with primer set P1-P3 as the binding site for primer-P3 is located in the deleted region. The sample with heterozygous mutation of Δ619 allele, will generate two different sized products: 1671bp with primer sets P1-P5 for normal allele and a product of 1052bp for mutant allele, if the same sample is amplified by primers P4-P5 it will produce 1212bp for normal allele and 593bp product for mutant allele, product of primer pair P1-P3 will be of 1457bp. Sample with wild type allele will produce 1671bp product with primer P1-P5, 1457bp product with primers P1-P3 and 1212bp product with primers P4-P5. The primers P4-P5 were used for reconfirmation of the results generated by P1-P5 and P1-P3 primer sets. To simplify the understanding of the PCR analysis, all the results with primer combinations is provided in the Table 2.

S.No.	Sample with alleles	Primer pairs	PCR Product size
1	For all kinds of samples (samples with unaffected wild type alleles, heterozygous mutant alleles and affected mutant alleles)	P1-P2	696bp
2	For all kinds of samples (samples with unaffected wild type alleles, heterozygous mutant alleles and affected mutant alleles)	P4-P6	719bp
3	Sample with homozygous mutant Δ619 allele	P1-P5	1052bp
		P4-P5	593bp
		P1-P3	No amplification
4	Sample with heterozygous mutation of Δ619 allele	P1-P5	1671bp and 1052bp
		P4-P5	1212bp and 593bp
		P4-P5	1457
5	Sample with wild type of Δ619 allele	P1-P3	1457bp
		P4-P5	P4-P5
		P1-P5	1671bp

Table 2: Amplicon details and gap PCR analysis.

As per Chan et al. [15], two primer pairs P1-P2 and P4-P6 were designed to detect all the mutations via sequencing approach, but in present study all the mutations were covered by the primers P1-P2. Amplicon generated by primer pairs P1-P2 has the size of 696bp covering exon-1, intervening sequence-I (IVS-I) and exon-2 (Figure 1) whereas amplicon generated by primer pairs P4-P6 has the size of 719bp and it covers part of IVS-II and entire exon-3. It is worth noting that, most prevalent mutations of *HBB* gene for Indian population is covered by primers P1-P2, however in our study we also sequenced the amplicons generated using P4-P6 primer set to confirm the presence of

any novel mutations before concluding the sample as negative for β-thalassemia assay.

In present study, 11 mutations of *HBB* gene were detected including five point mutations *HBB*.C.92+5 G>C, HBB:c.47G>A, HBB:c.92G>C, HBB:c.92+1G>T, HBB:c.-50A>C one insertion *HBB*:c.27_28insG and two deleterious mutations including major Δ619bp deletion and HBB:c.124_127delTTCT and three sickle cell mutations c. 20A>T,p.E6V, c.19G>A,p.E6K and c.79G>A,p.E26K. Demographic details of the samples as well as mutations identified by the test were summarized in Table 3 for all the family/trio samples. Frequency of the detected mutations is presented in the pie chart (Figure 4). The results presented in the pie chart indicates that the HBB.C.92+5 G>C is most prevalent mutation (44.93%), followed by HBB.C.20 A>T (14.49%), whereas HBB.C.-50 A>C, HBB.C.-138 C>T (-88 C>T), HBB.C.92 G>C (Codon 30) mutations were the least prevalent (1.45%). The results are presented with the classification of β^+ and β^0 mutations. All four possible types of thalassemia mutations i.e., ββ, $\beta^+\beta^+$, $\beta^+\beta^0$, $\beta^0\beta^0$ are found in present study representing the presence of variety of mutations in the population. In case of family studies, trio samples were taken which incorporates amniotic fluid or CVS sample along with both parents samples. Out of 21 trio samples studied, 19 families were from Rajkot, Gujarat whereas two families were from Dhaka, Bangladesh. Out of 138 samples, 12 samples have been identified with compound heterozygosity with different combinations of mutations leading to thalassemia intermedia to thalassemia major. All the AF and CVS samples from the trio samples were checked for any maternal contamination using the method described by Batanian et al. [16]. In present study, all the samples subjected to MCC check confirmed absence of any maternal contamination (data not provided).

S.No.	Sample Type	Sample Details	Mutation	Genotype	Test Result
Family 1	EDTA Blood	Father	HBB.C.47 G>A	$\beta^0\beta$ (GA)	Thalassemia Minor
	EDTA Blood	Mother	HBB.C.47 G>A	$\beta^0\beta$(GA)	Thalassemia Minor
	Amniotic Fluid	Foetus	HBB.C.47 G>A	$\beta^0\beta^0$(AA)	Thalassemia Major
Family 2	EDTA Blood	Father	HBB.C.92+5 G>C	$\beta^0\beta$(GC)	Thalassemia Minor
	EDTA Blood	Mother	HBB.C.92+5 G>C	$\beta^0\beta$(GC)	Thalassemia Minor
	Amniotic Fluid	Foetus	HBB.C.92+5 G>C	$\beta^0\beta^0$(CC)	Thalassemia Major
Family 3	EDTA Blood	Mother	HBB.C.92+5 G>C	$\beta^0\beta$(GC)	Thalassemia Minor
	EDTA Blood	Father	HBB.C.92+5 G>C	$\beta^0\beta$(GC)	Thalassemia Minor
	Amniotic Fluid	Foetus	HBB.C.92+5 G>C	$\beta^0\beta^0$(CC)	Thalassemia Major
Family 4	EDTA Blood	Father	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	EDTA Blood	Mother	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	Amniotic Fluid	Foetus	HBB.C.92+5 G>C	β^+ β^+(CC)	Thalassemia Major
Family 5	Heparin Blood	Father	Δ619 Deletion	$\beta^0\beta$	Thalassemia Minor
	Heparin Blood	Mother	HBB.C.92+1 G>T	$\beta^0\beta$	Thalassemia Minor
Family 6	EDTA Blood	Father	None	NA	No Beta-Thalassemia
			C.79 G>A, p.E26K	Hb E (Heterozygous)	Hb E Sickle Cell Anemia trait
	EDTA Blood	Mother	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	Amniotic Fluid	Foetus	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
Family 7	EDTA Blood	Father	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	EDTA Blood	Mother	None	NA	No Beta-Thalassemia
			C.79 G>A, p.E26K	Hb E Heterozygous	Hb E Sickle Cell Anemia trait
	CVS	Foetus	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
			C.79 G>A, p.E26K	Hb E Heterozygous	Hb E Sickle Cell Anemia trait
Family 8	EDTA Blood	Father	HBB.C.124_127delTTCT	β^+ β(Heterozygous)	Thalassemia Minor

	EDTA Blood	Mother	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	Amniotic Fluid	Foetus	HBB.C.124_127delTTCT	β^+ β(Heterozygous)	Thalassemia Minor
Family 9	EDTA Blood	Mother	HBB.C.124_127delTTCT (Codon 41/42 - TTCT)	β^+ β(Heterozygous)	Thalassemia Minor
	EDTA Blood	Father	HBB.C.27_28insG (Codon 8/9 (+G))	β°β(Heterozygous)	Thalassemia Minor
	CVS	Foetus	None	ββ	Normal
Family 10	EDTA Blood	Mother	HBB.C.92+1 G>T (IVS I-1(G>T))	β°β(Heterozygous)	Thalassemia Minor
	EDTA Blood	Father	Δ619 Deletion	β°β(Heterozygous)	Thalassemia Minor
	CVS	Foetus	None	ββ	Normal
Family 11	EDTA Blood	Father	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	EDTA Blood	Mother	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	EDTA Blood	Mother's mother	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	EDTA Blood	Mother's father	HBB.C.92+5 G>C	ββ(GG)	Normal
	EDTA Blood	Mother's sister	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	Swab sample	Son	HBB.C.92+5 G>C	$\beta^+\beta^+$(CC)	Thalassemia Major
Family 12	EDTA Blood	Father	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	EDTA Blood	Mother	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	Amniotic Fluid	Foetus	HBB.C.92+5 G>C	β^+ β^+(CC)	Thalassemia Major
Family 13	EDTA Blood	Father	HBB.C.47 G>A	β^0β(GA)	Thalassemia Minor
	EDTA Blood	Mother	HBB.C.47 G>A	β^0β(GA)	Thalassemia Minor
	Amniotic Fluid	Foetus	HBB.C.47 G>A	$\beta^0\beta^0$(AA)	Thalassemia Major
Family 14	EDTA Blood	Father	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	EDTA Blood	Mother	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	Amniotic Fluid	Foetus	HBB.C.92+5 G>C	β^+ β^+(CC)	Thalassemia Major
Family 15	EDTA Blood	Father	HBB.C.47 G>A	β^0β(GA)	Thalassemia Minor
	EDTA Blood	Mother	HBB.C.47 G>A	β^0β(GA)	Thalassemia Minor
	Amniotic Fluid	Foetus	None	ββ	Normal
Family 16	EDTA Blood	Father	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	EDTA Blood	Mother	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	Amniotic Fluid	Foetus	None	ββ	Normal
Family 17	EDTA Blood	Father	HBB.C.-50 A>C	β^+ β(AC)	Thalassemia Minor
	EDTA Blood	Mother	None	ββ	Normal
	Amniotic Fluid	Foetus	None	ββ	Normal
Family 18	EDTA Blood	Father	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	EDTA Blood	Mother	HBB.C.124_127delTTCT	β^+ β	Thalassemia Minor
	Amniotic Fluid	Foetus	HBB.C.124_127delTTCT and	$\beta^+\beta^+$	Thalassemia Major
			HBB.C.92+5 G>C	(TTCT het + GC)	

Family 19	EDTA Blood	Father	HBB.C.47 G>A	$\beta^0\beta$(GA)	Thalassemia Minor
	EDTA Blood	Mother	HBB.C.47 G>A	$\beta^0\beta$(GA)	Thalassemia Minor
	Amniotic Fluid	Foetus 1	HBB.C.47 G>A	$\beta^0\beta^0$(AA)	Thalassemia Major
		Foetus 2	None	ββ	Normal
Family 20	EDTA Blood	Father	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	EDTA Blood	Mother	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	Amniotic Fluid	Foetus	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
Family 21	EDTA Blood	Father	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	EDTA Blood	Mother	HBB.C.92+5 G>C	β^+ β(GC)	Thalassemia Minor
	Amniotic Fluid	Foetus	None	ββ	Normal

Table 3: Details of samples for prenatal beta thalassemia mutation testing.

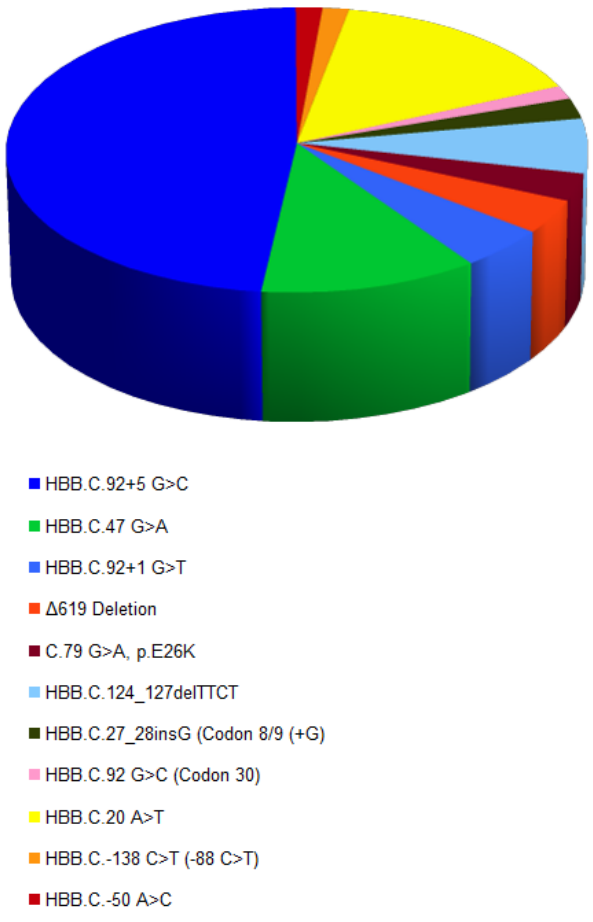

Figure 4: Pictorial representation of mutation frequencies detected in 138 specimens.

Discussion and Conclusion

As per Census of 2011, population size of India is 1.21 billion. With the increasing population, it is very important to investigate allele and gene frequencies of genetic or inherited disorders, including thalassemias (both alpha and beta thalassemia). Indian population includes 427 tribal groups and 4693 endogamous communities, making it more prone to genetic diseases because of less diversified gene pool [3]. Several studies on hemoglobinopathies and thalassemia have been conducted in various states and population of India by using chromatography based methods [16-19]. Occurance of thalassemia in India is up to 3%, but in some communities like Sindhis, Punjabis, Lohanas, Kutchi Bhanushalis, Jains and Bohris have a higher prevalence 4-17% [3,8,18,20-24]. These chromatography based methods can be used to detect hemoglobinopathies in the blood samples but not for detecting prenatal cases. Prenatal testing requires more sensitive and DNA based methods like probe based detection using real time polymerase chain reaction (PCR), amplification refractory mutation system (ARMS) based method, sanger sequencing based method etc.

American College of Medical Genetics (http://www.acmg.net/Pages/ACMG_Activities/stds-2002/g.htm, last accessed August 15, 2014), the Clinical Laboratory Standards Institute (2006 edition of Standards and Guidelines for Clinical Genetics Laboratories, prenatal testing section G19 first added in 2003; Molecular Diagnostic Methods for Genetic Diseases, Approved Guideline - Second Edition, MM1-A2 Vol 26 No 27), and the Clinical Molecular Genetics Society in the UK have incorporated standards and guidelines for cytogenetic and molecular genetic testing that recommend MCC testing in prenatal diagnosis [23]. The presence of MCC in fetal samples can lead to misdiagnosis when using molecular techniques to detect pathogenic variations. In order to provide the correct prenatal results, the possibility of the presence of contaminating maternal or co-fetal material in AF or CVS sample should be ruled out [24]. In present study, we have used the method described by Batanian et al. to detect MCC in the fetal sample [16]. Batanian et al. used combination of two non-radio labeled primer sets APOB and YNZ22 to solve the purpose, out of which APOB primer sets provided good results in our study hence we used APOB VNTR primers to screen out MCC [16]. To differentiate between contaminated and pure AF/CVS samples, we have spiked 10% of

maternal DNA to the AF DNA during PCR reaction set up and analyzed them on 2% agarose gel (Figure 3). In this image, the AF spiked with maternal sample is showing extra band which is originally present in maternal sample. This concludes that the presented APOB VNTR- gel based method can be used to detect maternal contamination in the provided AF/CVS samples.

High performance liquid chromatography (HPLC) is commonly used method for detecting β-thalassemia mutations which targets the abnormality in the size and structure of blood cells. It is economically very useful method for primary screening but it cannot detect prenatal β-thalassemia as in such case blood will not be available as sample type. PCR based methods are second option for detecting the β-thalassemia mutations but it will be again restricted to known and targeted mutations only hence a method which can overcome the limitations of both the aforesaid methods was required. In present study, we have developed a method by modifying the primers and protocols described by Chan et al. [15] for detecting β-Globin mutations by spanning the complete gene by sequencing method. As whole gene sequencing is done in this method, all the novel and unknown mutations can also be identified. Moreover, sickle cell anemia related mutations are also covered in the β-globin gene so that the cases of compound heterozygosity of sickle cell with β-thalassemia can also be identified which is frequently observed in northern and eastern Indian population [25].

Out of more than 200 mutations reported for β-thalassemia worldwide [14], mutations included in this study covers 93.5% of Indian populations [26]. Mutation HBB.C.92+5 G>C was found to be most common with the frequency of 44.54% for all the samples included in the study (Individual and trio samples also). Frequency of this mutation was found to be 44.8%, 49.8%, 67.9%, 50.7% and 71.4% in north, central, south, east and western Indian population respectively [26]. Frequency of all the mutations found in this study is presented by pie chart in Figure 4.

Total of 21 families screened in present study, out of which 19 families were from India (Gujarat) and rest two families were from Bangladesh (Dhaka). As *HBB* gene is the single gene responsible for Beta thalassemia [27], all variations whether related to thalassemia or sickle cell anemia, lead to symptoms of hemoglobinopathies. β-thalassemia follows an autosomal recessive pattern of inheritance, there are 25% chances of being β-thalassemia major, 25% chances of being normal and 50% chances of being β-thalassemia minor. In our study we came across 21 trio samples in which we observed 70.3% major where as 15.7% minor and 14% normal inheritance pattern in AF/CVS samples.

Out of 21 trio samples screened, one of the amniotic fluid sample from a family study was identified with compound heterozygosity of sickle cell mutation as well as beta thalassemia mutation, developing severe symptoms in the patient, similar to beta thalassemia major, similar compound heterozygosity was observed with three individual samples also [28]. Individual with single mutation in heterozygous state can be non-symptomatic or normal but patient with compound heterozygous mutation in case of beta thalassemia exhibits symptoms similar to thalassemia major. In the present study, we observed CVS samples from twins showing different mutations for *HBB* gene. Two CVS samples along with parents blood was provided for beta thalassemia investigation. Both parents were detected to be heterozygous for *HBB*.C.47 G>A mutation. In this case, one CVS sample was carrier and another CVS sample was detected to be beta thalassemia major. It is the typical case of segregation distortion, if twins are monozygotic in nature and rare event of chromosomal segregation of mutations, if twins are dizygotic (fraternal twins) in nature [29,30]. Further genetic analysis to differentiate twins were out of scope of this study.

In another case of the trio samples, we found fetus sample was having heterozygous genotype for two different *HBB* gene mutations HBB.C.92+5 G>C and HBB:c.92G>C being a thalassemia major case. This case was of the third pregnancy, earlier to this the family had two children, elder one was carrying same compound heterozygous mutations whereas younger child was thalassemia minor. Family history provided by the patients during the counseling suggested that the first child was having the symptoms similar thalassemia major, so their current pregnancy will also be thalassemia major because of the same genotype [31].

In our study we found 12 mutations. Present study comprises total of 138 samples including 66 samples from 21 families and 72 individual samples resulted in prenatal screening resulted as the most effective strategy in controlling β-thalassemia in few countries like Cyprus, Italy, Greece , UK etc. [32-35]. We strongly recommend that after primary screening of the parents, prenatal sample should be tested using sequencing based methods to avoid any false negative cases. Premarital screening followed by prenatal diagnosis can be a very good approach for thalassemia control in the developing countries like India.

Despite of medical advancements, there is still no cure for this disease, which makes it a threat to human beings. Regular blood transfusions and iron chelation therapy is required to increase the life; however this is not the complete cure for the disease [36]. Efficient way to deal with thalassemia is to prevent it by increasing awareness for premarital thalassemia testing followed by prenatal thalassemia testing in high risk communities and ethnic population.

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Disclaimers

Xcelris not liable for Patents or IPRs of Primer sequences are belonging to respective Researchers.

Conflict of Interest

The authors declare that they have no conflict of interest.

Note

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