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Biochemistry

EVALUATION OF MULTIPLEX ARMS PCR FOR SCREENING OF β-THALASSEMIA MUTATIONS

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ABSTRACT

Introduction: β-Thalassemia is one of the most common single gene disorder worldwide, which is caused by mutations in the β globin (HBB) gene on chromosome 11. In India, the prevalence of β-Thalassemia is 2-3% in general population and as high as 17% in high risk communities.

Objective: To evaluate Multiplex ARMS PCR for faster detection of β-Thalassemia mutation.

Methods: ARMS PCR approach identifying 5 common β-globin gene mutations were designed and employed to investigate 30 β-thalassaemia carriers. Two β-Thalassemia mutations [IVS I-5(G-C) and Codon 8/9(+G)] were analysed using Multiplex ARMS PCR in a single reaction.

Result: With the help of Multiplex ARMS PCR two β-Thalassemia mutations [IVS I-5(G-C) and Codon 8/9(+G)] were identified in a single reaction.

Conclusion: Multiplex ARMS PCR is accurate, cost-effective, faster and labour efficient. Hence it should find a place in the routine detection of mutations of β-globin gene which can accelerate identification by mutational analysis.

KEYWORDS : multiplex arms pcr, β thalassemia

INTRODUCTION:

β-Thalassemia is one of the most common single gene disorder worldwide, which is caused by mutations in the β globin (HBB) gene on chromosome 11.(1) β-Thalassemia is a highly prevalent autosomal recessive disorder characterized by the reduced or absent expression of the β-globin gene, leading to an imbalance of α and β-globin chains. β-thalassemia is a quantitative deficiency of β-globin production and is usually due to DNA mutations of the β-globin gene cluster and result from mutations affecting gene transcription, RNA processing, alter splice junctions or splice consensus sequences, mutations within exons and introns that create an alternative splice site.(2) Majority of the mutations are point mutations and unlike α-thalassemia deletion mutations are relatively less frequent. In India, the prevalence of Thalassemia is 2-3% in general population and as high as 17% in high risk communities.(3) >200 mutations are responsible for β-Thalassemia,(4) of which 5 common mutations, prevailing in Western Maharashtra are considered in the study. In general, each population has a unique spectrum of β-thalassemia mutations, consisting of a few very common mutations and a variable number of rare mutations. A technique that can simultaneously analyse a sample for the presence of multiple mutations would therefore be very useful.

MATERIALS AND METHODS:

The Cross-sectional study was conducted in B.J Medical College and Sassoon General Hospital, Pune. Institutional Ethical Committee permission was obtained prior to commencement of the study. Parents of known β-Thalassemia Major patients with their HbA2 >3.5% were included. Other family members who are carrier and parents associated with other hemoglobinopathies were excluded. A total of 30 carrier subjects were randomly selected. After informed consent was obtained from each individual, six millilitre of whole blood samples were collected in EDTA bottle.

DNA extraction from whole blood, was done by 'salting out method' as described by Helms C.(5) Mutational analysis was carried out using the amplification refractory mutation system polymerase chain reaction (ARMS-PCR) . The mutation-specific ARMS primers, control primers, and common primers used to diagnose the five common β-thalassemia mutations in this study are listed in Table 1. For each reaction there were total four primers used, two of these four primers serve as internal control (Control C and control D primers) which amplifies a part of β-globin gene at the 3′ end. These internal control primers also encompasses the 619 bp deletion common in the Indian Subcontinent, if the 619 bp deletion mutation is present it forms 242 bp PCR product.(6)

Table no.1: For the present study, we used the primer sequences as described by J.M Old et al (7)

S.No	Primers	Primer sequence (5'-3')
1	Common A	CCCCTTCCTATGACATGAACTTAA
2	Common B	ACCTCACCCTGTGGAGCCAC
3	Control C	GAGTCAAGGCTGAGAGATGCAGGA
4	Control D	CAATGTATCATGCCTCTTTGCACC
5	IVS I-5(G-C)	CTCCTTAAACCTGTCTTGTAACCTTGTTAG
6	IVS I-1(G-A)	TTAAACCTGTCTTGTAACCTTGATACCGAT
7	Codon 8/9 (+G)	CCTTGCCCCACAGGGCAGTAACGGCACACC
8	Codon 15 (G-A)	TGAGGAGAAGTCTGCCGTTACTGCCCAGTA

The PCR was carried out in a total volume of 10 µl of reaction mixture, containing 0.1 to 0.5 µg of genomic DNA, 0.2 µM of primer mix, 1.5 mM MgCl2; 200 µM each deoxynucleotides triphosphates, and 0.5 U/µl Taq DNA polymerase.(7) Cycling was carried out on the thermal cycler with an initial 5 min denaturation step at 95 °C, followed by 30 cycles at 94 °C for 30s, 30s at 65 °C annealing temperature, and 72 °C for 1 min 30 s and the final extension at 72 °C for 5 min. Following amplification, 5 µl aliquots were mixed with gel loading buffer and electrophoresed on 2 % agarose gel in Tris--acetate–EDTA buffer for 1 h at 5-8 volts/cm, gel was stained with ethidium bromide and visualized using UV trans-illuminator.

Table no.2: Product of ARMS PCR reactions using various primers and their interpretation for combination of primers

Mutations	Primer Combination	PCR Product Size(bp)	Interpretation
IVS I-5(G-C)	2+3+4+5	861+285	IVS I-5 mutation present
		861	IVS I-5 mutation absent
		861+242	Heterozygous for 619 bp deletion
		242	Homozygous for 619 bp deletion
IVS I-1(G-A)	2+3+4+6	861+281	IVS I-1 mutation present
		861	IVS I-1 mutation absent
Codon 8/9 (+G)	2+3+4+7	861+225	Codon 8/9 mutation present
		861	Codon 8/9 mutation absent
Codon 15(G-A)	1+3+4+8	861+500	Codon 15 mutation present
		861	Codon 15 mutation absent

Multiplex Amplification Refractory Mutation System Polymerase Chain Reaction:

With the use of Multiplex ARMS PCR, more than one target sequence can be amplified by using multiple primer pairs in a single reaction mixture. Amplification refractory mutation system (ARMS) has been widely used for identification of β-Thalassemia mutations but it usually detects one mutation per reaction and can be laborious and expensive.(7) To overcome this problem, in the present study, multiplex PCR protocols using Multiplex ARMS-PCR have been evaluated for β-Thalassemia detection.

We evaluated Multiplex ARMS PCR for two common mutations [IVS I-5(G-C) and codon 8/9(+G)]

Primers included for the study:

1) Common B Primer
2) Control C Primer
3) Control D Primer
4) Primer for IVS I-5(G-C) mutation
5) Primer for codon 8/9(+G) mutation

For one PCR reaction, a mixture was prepared containing master mix, the above primer mix, and DNA samples positive for IVS I-5 and Codon 8/9 mutations. For multiplexing varying concentration of mutant primers (0.1µM, 0.2µM, and 0.3µM) were tested.

Table no.3: Varying concentrations of mutant primers used in this study

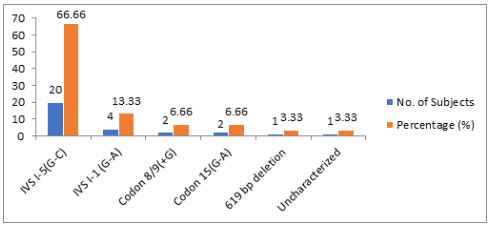
Reaction	Concentration of Primers B, C, D each	Concentration of Mutant Primers for IVS I-5 and Codon 8/9 each
Reaction 1	0.2 µM	0.1 µM
Reaction 2	0.2 µM	0.2 µM
Reaction 3	0.2 µM	0.3 µM

Reactions were analysed with two different annealing temperature 65°C and 66°C Further Post PCR analysis was done by agarose gel electrophoresis.

OBSERVATION AND RESULTS:

Mutational analysis was performed on 30 subjects who were proven carriers for β-Thalassemia (parents of known β-Thalassemia major patients). We studied five common mutations from Western India on DNA samples using PCR based techniques (ARMS). The G-C substitution at IVSI-5, a mutation of Asian-Indian origin, is the most common mutation reported in this study. Out of 30 subjects of β-Thalassemia trait, a total of 20 (66.66 %) subjects were reported as IVS I-5 (G–C) mutation, IVS I-1(G–A) of lower frequency was found in 4 (13.33 %) subjects. Codon 8/9 (+G) was found in 2 subjects (6.66 %). Codon 15 (G–A) was found in another 2(6.66 %) subjects. 619 bp deletion of Indian origin was found in 1 subject (3.33%). There was 1 (3.33%) subject who did not show any of the five mutations studied.

Fig 1: Graphical presentation of the five common mutations of β-thalassemia carrier subjects



Our purpose was also to evaluate single-tube multiplexed PCR assays using DNA specimens to identify the mutations responsible for β-Thalassemia in order to expedite faster diagnosis. This multiplex ARMS approach was standardized using DNA samples with known mutations for β-Thalassemia. We identified two β-Thalassemia mutations [IVS I-5(G-C) and Codon 8/9(+G)] in multiplexed ARMS PCR in single reaction from samples positive for these mutations.

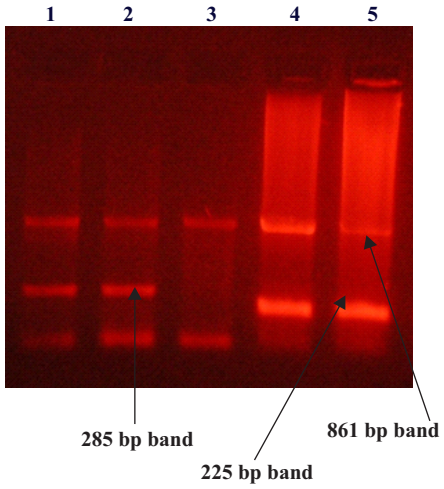
Table no.4: Result of the products obtained at various concentrations of mutation specific primers

Sl. No.	Test DNA	Products size In Varying Concentration of Mutant Primers (in µM)		
		0.1	0.2	0.3
1	Wild Type	861 bp band of DNA obtained	861 bp	861+ Non-Specific bands
2	Carrier for IVC I-5 (G-C)	861+285	861+285	861+285+ weak bands of 225
3	Carrier for codon 8/9(+G)	861+225	861+225	861+225+ weak bands of 285

We found out that with 0.3µM concentration there was non-specific amplification.

But with 0.1µM concentration, amplification was specific and sufficient and appropriate result was obtained.

Fig 2: Photograph showing amplification of IVS I 5 mutant and Codon 8/9 using combination of mutant primers in the same reaction mix.



Lane 1 &2: DNA Sample carrier for IVS I 5 mutation
Lane 3: DNA sample Negative for both IVS I 5 and Codon 8/9 mutation
Lane 4 & 5: DNA Sample carrier for Codon 8/9 mutation

CONCLUSION:

Since β-globin gene mutations are responsible for β-Thalassemia disease, detection of these particular genetic mutations is important. Characterization of the mutational pattern revealed from our study will provide data for appropriate genetic counselling and prenatal work up to the geneticist.

In this study, there was successful development of Multiplex ARMS PCR (more than 1 number of mutations detected in a single PCR reaction excluding 619 bp deletion) to identify common β-Thalassemia mutations. The results obtained from standard ARMS Protocol and Multiplex ARMS PCR which we have developed was equivocal. This clearly confirms the equivalence of the methods for the mutations studied. Thus, the major advantage of Multiplex ARMS PCR is that this assay is accurate, cost-effective, faster, labour efficient, reliable, non-isotopic and provides a within-assay quality control for the exclusion of false-negative results. Hence it should find a place in the routine detection of mutations of β-globin gene causing β-Thalassemia in India, after evaluating utility of multiplexing with other mutant primers.

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