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A sensitive TaqMan PCR-based assay for screening of Sickle cell anemia and its carriers in a population

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ABSTRACT

Sickle cell anemia is a genetic disorder where the red blood cells alter their shape due to the polymerization in hemoglobin chains thus disrupting their ability to circulate through the blood vessels. The disease is caused by a 'A' to 'T' base substitution at a specific position in the HBB gene. Whereas different methods of detection of sickle cell hemoglobin in blood are available, we propose a TaqMan PCR-based sensitive genetic testing assay for the early detection of the sickle cell allele in diseased individuals as well as identification of carriers in a population.

I. INTRODUCTION

Adult human hemoglobin molecule is composed of two alpha globin chains and two beta globin chains, with an associated heme group. Alpha globin is coded by two genes HBA1 and HBA2 (produced by a gene duplication event) residing on chromosome 16, while beta globin is coded by HBB gene on chromosome 11. Sickle cell anemia (SCA) is a disease where the RBCs become sickle-shaped obstructing blood flow through narrow arteries and veins. This happens due to a single-nucleotide polymorphism (SNP) in the HBB gene encoding beta globin. The sixth codon of HBB gene mutates from GAG to GTG leading to a substitution of the glutamate residue in the sixth position of the beta chain by a valine residue (1). This disrupts the electrostatic interaction between the sixth amino acid and N-terminal valine resulting in hemoglobin molecules that stick to one another which ultimately causes RBCs to become crescent-shaped and sticky leading to their early death and obstruction of blood vessels (2).This disease is very common in countries of sub-Saharan Africa. In India, sickle cell anemia mostly prevails among the tribal population in the Nilgiris (3).

Sickle cell anemia is usually tested by solubility test and hemoglobin electrophoresis to detect sickle cell hemoglobin (HbS) (4,5). Genetic screening would be the best means for early detection of SCA in fetal blood and chorionic-villus samples (6). DNA sequencing is an expensive and a time-consuming method. Besides, it cannot readily distinguish between homozygous mutants (HbS/HbS) and heterozygous carriers (HbA/HbS). TaqMan Polymerase chain reaction (PCR) is a simple, fast, highly specific and highly sensitive means for disease diagnosis, and has currently been widely adopted in molecular testing including SNP detection (7). We have developed a simple TaqMan PCR based SCA detection method targeting the GAG to GTG SNP that can detect the presence of HbS allele and discriminate homozygous HbSmutants from carrier heterozygotes with the sickle-cell trait.

II. RESULTS AND DISCUSSIONS

Design of Primers and Probes

Hemoglobin gene beta chain (HBB) DNA sequences retrieved from NCBI database were aligned using Clustal-Omega algorithm (Fig 1a). Primers were designed spanning the sixth codon and the dual-labeled TaqMan probe targeted the 'A' to 'G' mutation at the sixth codon as shown in Fig 1b.

Generation of SCA wild-type and mutant clones

A 105-bp region at the 5'-end of HBB gene was amplified from genomic DNA isolated from five normal human blood samples (Fig 2a). An amplicon of desired size was cloned into pGEM-T vector and transformed into E. coli Mach1 cells. Plasmids were isolated from positive clones (Fig 2b) and were confirmed by sequencing (Fig 2c). The mutant clone with 'A' to 'G' base transversion in the 17th position (corresponding with the sixth codon) was created by site-directed mutagnesis. Plasmids isolated from mutant clones were initially screened with PCR using T7-

Fwd/SP6-Rev as well as gene-specific primers (Fig 2c), and the position of the mutation was confirmed by sequencing (Fig 2d).

Determination of assay specificity

Multiplex TaqMan PCR reaction was developed for the detection of sickle cell anemia wild-type (HbA) and mutant alleles (HbS). The common forward and reverse primers along with the MUT probe (for mutant allele detection) gave amplification curves from mutant clones but no amplification from wild-type clones or control human DNA detected in HEX fluorescence channel (Fig 3a). On the contrary, the common primers among with the WT probe (for the detection of wild-type allele) gave amplification from wild-type clones and control human DNA, but did not amplify from mutant clones detected in FAM fluorescence channel (Fig 3b).

Assay sensitivity determination

The absolute sensitivity of the assay was measured using serial dilutions of the wild-type (HbA) and mutant (HbS) plasmid clones as a template for detection in TaqMan PCR. The PCR was run till 45 cycles and amplification till cut-off Ct value 40 was considered for Limit of Detection (LoD) determination. For both wild-type and mutant clone, the LoD of the assay was found to be 100 DNA copies per reaction (Fig 4).

Validation on Positive Samples

The multiplex TaqMan PCR assay for Sickle cell anemia detection was tested on 3 known positive blood samples previously validated by DNA sequencing. 10 ng DNA was used in the assay. Wild-type HbA allele was detected in the FAM fluorescence channel and the mutant HbS allele was detected in the HEX channel. Out of the 3 samples, 2 of them were found to be heterozygous for the sickle cell allele (HbA/HbS) (Fig 5a & 5b) and 1 was homozygous for sickle cell allele (HbS/HbS) (Fig 5c).

III. MATERIALS & METHODS:

Genomic DNA isolation from blood samples

Whole blood from five control humans was freshly collected in K2-EDTA vials. Genomic DNA was isolated from the blood samples using GSure Blood DNA Isolation kit (GCC Biotech) as per the manufacturer's protocol.

PCR conditions for HBB wild-type gene fragment

A 105-bp was amplified from the blood DNA. Per 25 µL reaction contained 100 ng of DNA template, 0.5 µM each of HBB Fwd and HBB Rev primers, 0.2 mM dNTP mix, 2.5 mM $MgCl_2$, 1U G9 Taq Polymerase (GCC Biotech) and 1X Taq reaction buffer. PCR was carried out at the following conditions: Initial denaturation at 95°C for 5 min; cycling at 95°C for 15 sec, 60°C for 15 sec and 72°C for 12 sec for 35 cycles; final extension at 72°C for 3 min; infinite hold at 4°C.

Cloning of HBB gene fragment in pGEM-T vector

The PCR amplicon run in gel was gel eluted and purified using GSure Gel extraction kit (GCC Biotech). Ligation was set up with insert: vector ratio 3:1. Ligation was performed overnight at 4°C. The ligated product was directly transformed into 50 uL ofE. coli Mach1 competent cells prepared in accordance with previously described protocols (8). Colonies appearing on overnight-grown plates were screened for the presence of insert of desired size.

Colony PCR conditions

A PCR mix was prepared with 1X (final conc.) of 2X G9 PCR Master mix (GCC Biotech) and 0.5 µM each of T7-Fwd and SP6-Rev Primers. Individual colonies were pricked with a microtip (0.5-10 µL) and mixed well into the PCR mix. PCR was run at the following cycling conditions:Cell lysis and Initial denaturation at 95°C for 15 min; cycling at 95°C for 15 sec, 60°C for 15 sec and 72°C for 30 sec for 40 cycles; final extension at 72°C for 5 min; infinite hold at 4°C.

Multiplex TaqMan PCR of HbA wild-type and HbS mutant alleles

TaqMan PCR was set up in 25 µL reaction volume using 13 µL of WRTaqMan master mix (GCC Biotech), 1 µL each of 10 µM HBB Fwd and HBB Rev primers, 1.5 µL each of 10 µM HbA WT Probe and HbS MUT Probe. TaqMan PCR was run under the following conditions: Initial denaturation at 95°C for 3 min; cycling at 95°C for 10 sec, 58°C for 30 sec for 40 cycles.

Table 1. List of primers & probes used in the assay

Sl. No.	Name of primer	Sequence (5' – 3')
1	HBB Fwd	ACAACTGTGTTCACTAGCAA
2	HBB Rev	CTTCATCCACGTTCACCTTG
3	SCA SDM Fwd	ACTCCTGTGGAGAAGTCTGCCGTTACTGC

4	SCA SDM Rev	TTCTCCACAGGAGTCAGGTGCACCATGGT
5	T7-Fwd	TAATACGACTCACTATAGGG
6	SP6 Rev	ATTTAGGTGACACTATAG
7	HbA WT Probe	FAM- GACTCCTGAGGAGAAGTC-BHQ1
8	HbS MUT Probe	HEX- GACTCCTGTGGAGAAGT-BHQ1

IV. CONCLUSION

A rapid TaqMan PCR based genetic testing of sickle cell anemia was developed for screening of individuals with sickle-cell phenotype and sickle-cell carrier in blood, amniotic fluid and CVS samples. The assay shows no cross-reactivity with unintended targets and the sensitivity of the assay is upto 10 DNA copies per reaction. The assay could detect sickle cell anemia positive samples and discriminate homozygous sickle-cell mutants (HbS/HbS) from heterozygous carriers (HbA/HbS).

Acknowledgement

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Conflict of Interest

The authors of the manuscript declare no conflict of interest.

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Figures:

Fig 1.

Fig 1. (a) Multiple sequence alignment of HBB gene N-terminal region and (b)location of primers & probes for SCA detection

Fig 2.

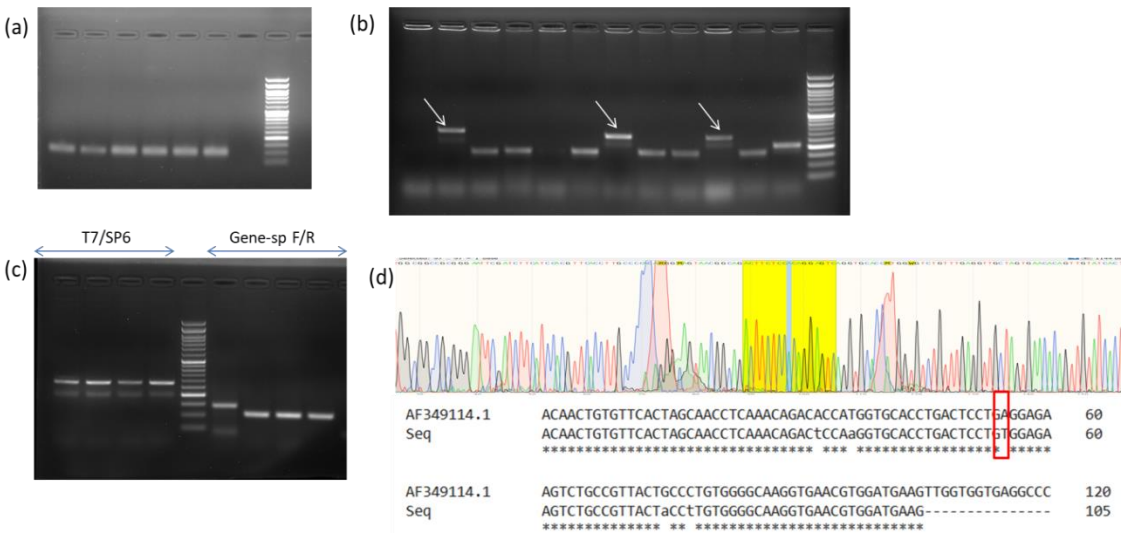

Fig 2 (a) Amplification of N-terminal region of HBB gene from 5 control human blood. Pdt size: 105-bp run along with 50-bp DNA ladder; (b) PCR results of 12 chosen colonies for colony pCR with T7 Fwd/SP6 Rev primers. Expected pdt size = 290-bp; DNA ladder used: 50-bp (c) Amplification from HbS mutant plasmid clones generated by site-directed mutagenesis using T7-Fwd/SP6-Rev primers (Pdt size: 290-bp) and gene-specific Fwd/Rev primers (Pdt size: 105-bp).

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]Fig 3.

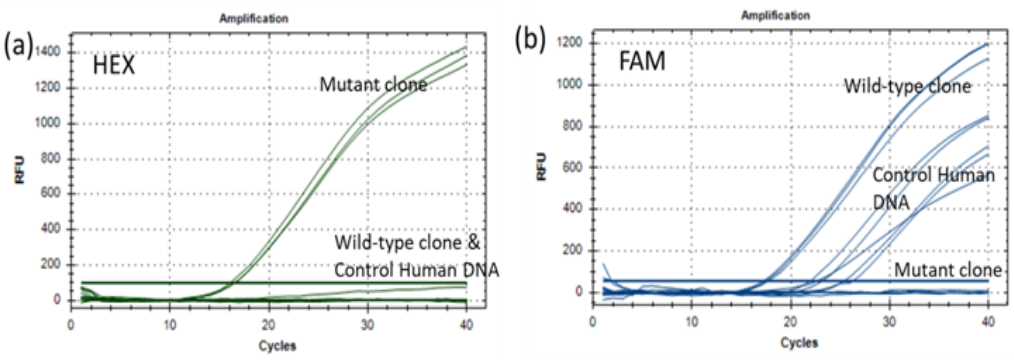

Fig 3. (a) TaqMan PCR amplification of HbA wild-type clone, HbS mutant clone and Control Human DNA with Wild-type allele-specific primers and probe; (b) Amplification of HbA wild-type clone, HbS mutant clone and Control Human DNA with sickle-cell mutant allele-specific primers and probe. Real-time PCR instrument: BioRad CFX-96

Fig 4.

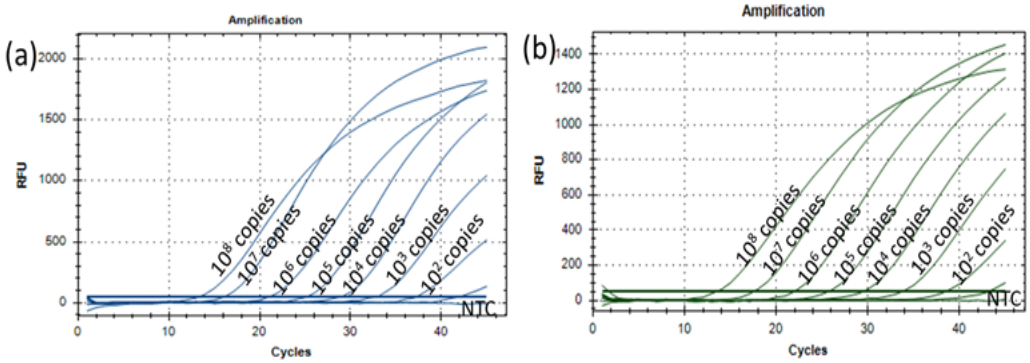

Fig 4. Amplification from serially diluted plasmid clones. (a) Wild-type HbA clone; (b) Mutant HbS clone; NTC: No-template control, Real-time PCR Instrument used: BioRad CFX-96

Fig 5.

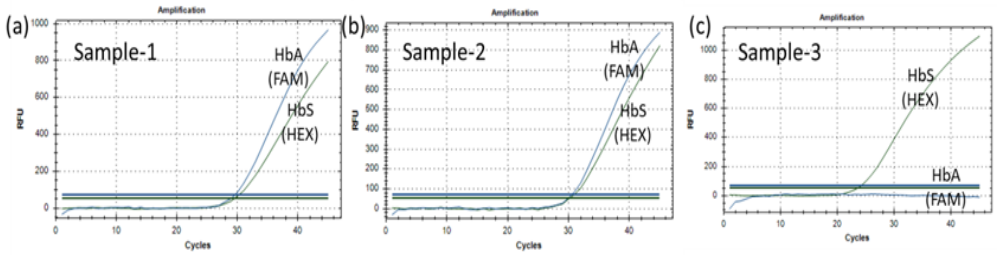

Fig 5. Multiplex TaqMan amplification from 3 sickle-cell anemia positive blood DNA