ORIGINAL RESEARCH PAPER

Microbiology

MULTIPLEX PCR BASED SINGLE NUCLEOTIDE POLYMORPHISMS ANALYSIS OF HBS POINT MUTATION

KEY WORDS: Sickle cell disease (SCD), Sickle hemoglobin (HbS), Single nucleotide polymorphism (SNP), Multiplex PCR

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ABSTRACT

Introduction: Sickle hemoglobin (HbS), an autosomal recessive hemoglobinopathy cause of Sickle cell disease (SCD), is widely sprayed around the globe affecting millions of people . SCD results from single nucleotide polymorphism (SNP) or point mutation causing amino acid substitution from Glutamic acid to Valine leads to sickled shape red blood cells. SNPs can be well studied by using allele-specific amplification (ASA) technique.
Aims & Objective: To develop a simple, rapid, easy and accurate genotyping method for SNP analysis of SCD.
Materials and methods: By performing different tests, a well characterized sample panel of 150 different types of samples was prepared. From this sample panel DNA was extracted and used for SNP-genotyping of SCD. Specific primers were used for performing monoplex PCR amplification of wild type allele (HbAA) and mutant allele (HbSS) were performed individually. By using the same primers multiplex PCR assay was experimented.
Results and conclusion: This is a simple and low cost molecular method for the detection of point mutation and useful tool for the diagnosis of SCD. The entire analysis can be performed in one reaction mixture, which results in higher speed, higher accuracy, and the need for smaller samples. This technique might be of great value for genotyping of homozygous sickle cell patients (SS) and heterozygous sickle cell trait (AS). But we found one discrepancy with double heterozygous (sickle β-thalassaemia) samples. We were not able to differentiate sickle cell carrier state (AS) from the double heterozygous like sickle β-thalassaemia state. So we conclude that for simultaneous detection of thalassaemia along with sickle cell requires addition of more primers specific for thalassaemia mutation. In addition to this when two bands, one for wild type allele and second for mutant allele appears, care must be taken to conclude whether the person is a sickle cell carrier (AS) or having double heterozygous (sickle β-thalassaemia) like condition.

INTRODUCTION:

Hemoglobinopathies are a variant group of inherited blood disorders that result from an alteration in the structure or synthesis of hemoglobin. More than a quarter of a million people are found to be born with one of the disorders in the world per year (Colah, Mukherjee, Martin, & Ghosh, 2015; Balgir, 2007). Various public health issues are shown as a result of these hemoglobinopathies, mainly in the Mediterranean area, in the Middle East and in parts of India, Africa and Southeast Asia. Among several types of hemoglobinopathies, particularly sickle hemoglobin (HbS) found in SCD is one of the most common of them (Sheikh *et al.,* 2017). A point mutation results in the replacement of hydrophilic Glutamic acid with the hydrophobic amino acid Valine at the sixth position (β^6 Glu to Val) in the β-globin amino acid chain. Clumping of the haemoglobin S (HbS) molecules into rigid fibers results in "sickling" of the entire red blood cells in the homozygous (HbSS) condition (Patra, Panigrahi & Banerjee, 2013; Valavi, Ansari & Zandian, 2010; Waye, & Chui, 2001).

Sickling test and haemoglobin solubility test are phenotypic screening techniques used to detect the presence of HbS. But these techniques were not 100% sensitive and specific and can not identify exact phenotype/genotype. Hemoglobin electrophoresis can determine the phenotype of SCD but it should not be performed on infants until they are at least 6 months old because of the presence of hemoglobin F (HbF) as the predominant haemoglobin at birth, or subjects who are recipients of allogeneic blood transfusion in the preceding three months. Thus all these primary screening tests are not enough to make proper diagnosis (Rajput, Naik & Italia, 2015).

In contrast to this, ASA by PCR is a commonly used technique for the detection of known SNPs, deletions, insertions and other variations in DNA sequence. Many effective methods are developed for the diagnosis of SNP like fluorescence resonance energy transfer, mass spectrophotometry or direct sequencing of PCR products. But execution of these techniques can require preparation of specific reagent and downstream purification, making them entangled and costly. However, assays based on PCR for SNP diagnosis have extensive potential in the clinical diagnostics because of its simplicity, potential minimal effort and low cost (Toye *et al.,* 2018; Ayatollahi & Haghshenas, 2004; Waterfall & Cobb, 2002).

A common ASA technique uses two complementary reactions, each containing a common primer, an allele-specific primer and Taq DNA polymerase enzyme lacking 3' to 5' proofreading activity. The first reaction contains a primer specific for the wild type or normal DNA sequence. In the same way, the second reaction contains a mutant specific primer unable to amplify wild-type DNA. Molecular confirmation is accomplished by investigation of the subsequent PCR amplicons. A normal individual (AA) will produce PCR amplicons only in the first reaction tube; a heterozygote (AS) amplifies products in both reaction tubes; and a homozygous (SS) individual will generate amplicons only in mutant specific reaction. It is necessary to run positive and negative internal controls for validating the PCR test results (Ali, 2018; Toye *et al.,* 2018; Ayatollahi & Haghshenas, 2004; Waterfall & Cobb, 2002).

The methodology for ASA by PCR described here is one of the fundamental and cost effective methods with high accuracy approach for diagnosis of SCD, where a small fragment of 517 bp can detect normal β-hemoglobin gene and 267 bp can

detect mutant allele. Using single tube PCR Based SNP-genotyping enables the early and accurate diagnosis of SCD.

MATERIALS AND METHODS:

This study was carried out in different steps as shown below. Data for all studies were collected scientifically for tabulation and analysis.

Collection of blood samples: Blood samples were collected from the patient came to the sickle cell department of Valsad Raktdan Kendra (VRK), a regional blood bank and hematological research centre, Valsad, Gujarat, India. Due approval of the project has been obtained from the scientific advisory committee and institutional ethical committee of VRK. Before collecting blood samples, a written consent was taken from the patient.

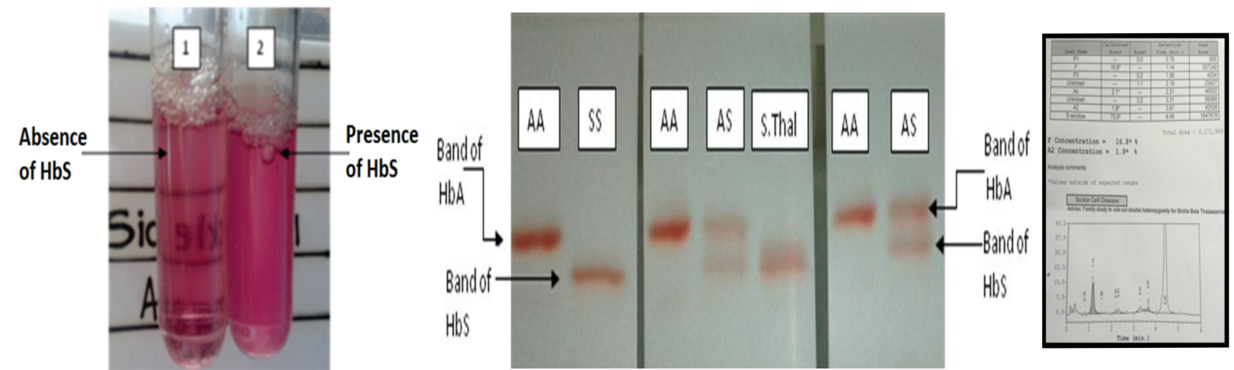
Characterization of samples:
To prepare a well characterized sample panel, below mentioned different tests were performed.

1. Complete hemogram:
2 mL whole blood was collected in EDTA anti-coagulant tube and labeled properly. Complete hemogram test was performed using calibrated sysmex pocH-100*i* particle counter to get hemoglobin concentration. Samples were characterized according to hemoglobin concentration.

2. Di-Thionite Tube (DTT) test as a primary screening test:
As a primary screening test for HbS all the samples were subjected to DTT test (Figure 1 a) (Rajput *et al.,* 2015).

***3. Haemoglobin electrophoresis*:**
For HbS confirmation all the samples were subjected to cellulose acetate hemoglobin electrophoresis. (Figure 1 b) (Mohanty & Colah, 2008).

4. High Performance Liquid Chromatography (HPLC):
A gold standard Hb HPLC (VARIANT β thalassaemia short program from Bio-Rad laboratories) was used to finalize the sickle status of blood samples (Figure 1 c) (Mohanty & Colah, 2008). Blood samples were stored at 2-8°C for further use.



a. Results of DTT test. 1. Normal blood sample (AA), 2. Sickle cell positive blood sample (AS/SS)

b. Cellulose acetate (Hb) electrophoresis test results

c. Variant HPLC result of SCD blood sample

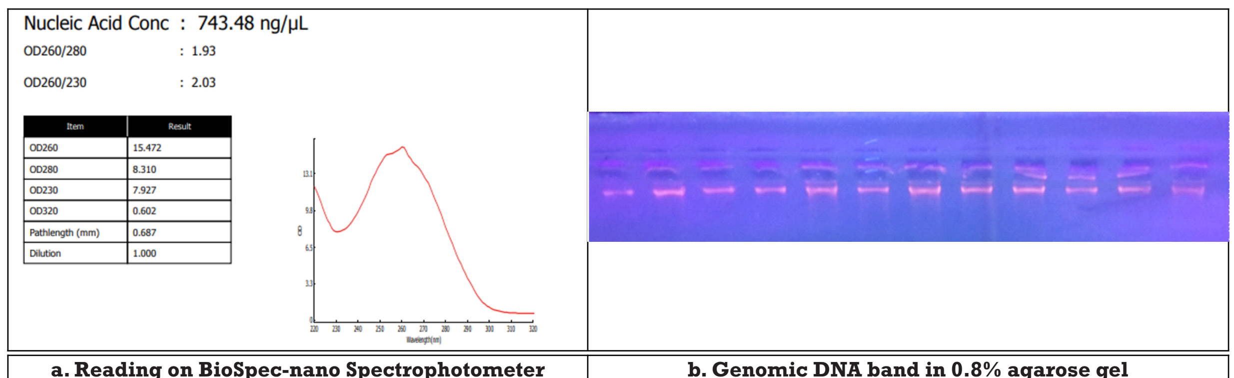
Figure 1: Results of different tests performed to prepare well characterized sample panel

Sample panel preparation: Based on the results of above mentioned different tests a well characterized sample panel was prepared as follows.

Table 1: Sample panel of different Hb Variants

Sr. No.	Sample Type	No. of sample
1	Normal controls (AA)	45
2	Sickle Cell Trait (AS)	50
3	Sickle Cell Anemia (SS)	40
4	Sickle β-Thalassaemia	5
5	β-Thalassaemia Minor	8
6	β-Thalassaemia Major	2
Total No. of Samples		**150**

Genomic DNA extraction: From all 150 samples genomic DNA was extracted using modified phenol-chloroform method of Mohanty & Colah *et al.,* 2008. The DNA concentration and purity was checked by taking O.D. at 260 and 280 nm on a BioSpec-nano Spectrophotometer (Shimadzu-Biotech) (Figure 2 a). DNA quality was further checked by electrophoresis on a 0.8% agarose gel (low EEO) containing EtBr at 80V for 30 minutes in 1X TBE buffer (pH 8.4). The gel was viewed on an ultraviolet trans-illuminator, a single band near the well indicating the presence of high molecular weight genomic DNA. Whereas the smear instead of a single band was a sign of sample degradation (Figure 2 b). DNA samples were stored at -20 °C for further use.



a. Reading on BioSpec-nano Spectrophotometer

b. Genomic DNA band in 0.8% agarose gel

Figure 2: Results of quality checking of extracted genomic DNA of sample panel

Reagents for ASA by PCR:

PCR reagents: Taq DNA Polymerase (Catalog No. D6677, 5 units/μL in 20 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, stabilizers, 50% glycerol), 10X PCR Buffer (Catalog No. P2192, 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl2 and 0.01% gelatin) were obtained from Sigma Aldrich, and dNTPs (10 mM; 2.5 mM each dATP, dCTP, dGTP, & dTTP) were procured from Saffron Lifescience.

Primers sequences: Following primers from the study of Waterfall and Cobb, (2001) were selected and synthesized from Eurofins Genomics India Pvt. Ltd. (Table 2). WT-AS and WT-CP517 were designed for amplification of a 517 bp fragment from the normal β-globin gene (wild type primer set). MUT-AS and MUT-CP267 were designed for amplification of a 267 bp fragment from homozygous mutant DNA (HbS/S) conferring SCD (mutant primer set).

Table 2: Primer sequences selected for ASA by PCR

Sr. No.	Oligonucleotide Name	Sequence 5' to 3'	Length
1	WT-AS	ATGGTGCACCTGACTCCTGA	20
2	WT-CP517	CCCCTTCCTATGACATGAACT	21
3	MUT-AS	CAGTAACGGCAGACTTCTCCA	21
4	MUT-CP267	GGGTTTGAAGTCCAACTCCTA	21

Monoplex PCR for ASA:

All reactions were performed in a final volume of 25 μL containing 10 ng total human genomic DNA template, 10X PCR reaction buffer, 1 U μL^{-1} Taq DNA polymerase enzyme, dNTPs and nuclease free water. Monoplex PCR were performed by using the wild type primer set and mutant primer set in separate PCR amplification tubes containing above reaction mixer. Amplification was performed using PCR Thermal Cycler (HiMedia Prima 96) and PCR conditions at initial denaturation (95°C for 2 minutes), followed by 30 cycles of denaturation (95°C for 30 seconds), primer annealing (60°C for 30 seconds), extension (72°C for 30 seconds) and final elongation (72°C for 10 minutes). PCR amplicons were analysed on 2% agarose gel and visualized by UV transillumination (Mohanty & Colah, 2008; Waterfall and Cobb, 2001).

Multiplex PCR for ASA:

After getting satisfactory results in Monoplex PCR, Multiplex PCR was experimented (Henegariu *et al.,* 1997). For finalizing Multiplex PCR different parameters as shown in table 3 were studied. Multiplex PCR was performed by adding wild type primer set and mutant primer set in a single PCR tube containing 10 ng total human genomic DNA template, 10X PCR reaction buffer, 1 U μL^{-1} Taq DNA polymerase enzyme, dNTPs and nuclease free water in final reaction volume 25 μL. Amplification was performed using PCR Thermal Cycler (HiMedia Prima 96).

Table 3: Optimized & Finalized parameters for Multiplex PCR

Optimized parameters		PCR Cycle	
1. Concentration of dNTPs (80 mM) 2. Concentration of Primers (0.8 μm) 3. Volume of DNA sample (10 ng) 4. Primer extension temperature 5. Number of Cycles 6. Monoplex then Multiplex	→	1. Initial denaturation at 95°C for 2 minutes 2. Denaturation at 95 °C for 30 seconds 3. Annealing at 60 °C for 30 seconds 4. Extension at 72 °C for 30 seconds 5. Final elongation at 72°C for 10 minutes 6. Hold at 4 °C	30 cycles (steps 2–4)

RESULTS:

Results of Monoplex PCR:

We have selected two primers, one for the wild type allele that amplifies 517 bp fragment from the normal β-globin gene, and 2nd for the mutant allele that amplifies 267 bp fragment from the sickle cell mutant β-globin gene. Both of these primers gave satisfactory results for normal (AA) type, sickle cell trait (AS) and sickle cell anemia (SS) types of samples. No discrepancies was seen in these types of samples. As shown in figure 3, Agarose gel electrophoresis results of PCR amplicons with HbSS revealed only 267 bp band and normal healthy (HbAA) individual revealed 517 bp band only.

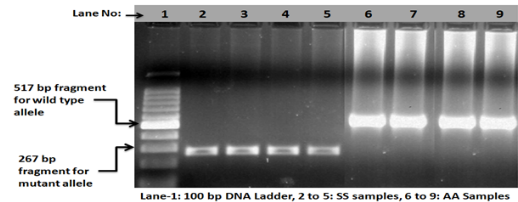


Figure 3: Results of monoplex PCR with wild type primer and mutant primer in individual reaction tubes.

Results of Multiplex PCR:

A total of 150 samples were analyzed in this study. 45 healthy normal individuals (HbAA), 40 patients with homozygous sickle cell anemia (HbSS), 50 sickle cell trait (HbAS), 5 double heterozygous (sickle β-thalassaemia), 8 β-thalassaemia minor, and 2 β-thalassaemia major were tested by multiplex PCR system.

Based on the results of PCR and gel electrophoresis, with HbSS showed only 267 bp band, healthy individual revealed 517 bp band only, while heterozygous revealed both 267 and 517 bp bands as indicated in figure 4.

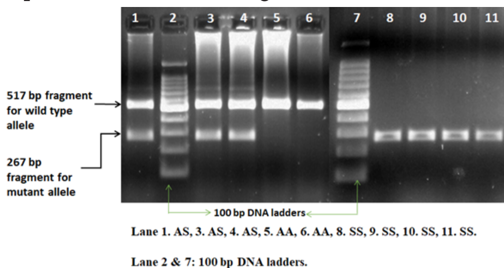


Figure 4: Results of Multiplex PCR with wild type primer and mutant primer in a single reaction tubes.

Here, genotyping was simply based on size discrimination of the resulting allele-specific PCR products, visualized by agarose gel electrophoresis. Results of Hb electrophoresis and PCR were in full agreement for sickle cell anemia, sickle cell trait, and healthy individuals.

But in the case of β-thalassaemia minor and β-thalassaemia major, only wild type 517 bp fragment was amplified (as shown in figure 5). This is because we have not incorporated any primer specific for detection of thalassaemia mutations. So gel electrophoresis results of PCR amplicons in case of β-thalassaemia minor and β-thalassaemia major revealed genotype of normal individuals.

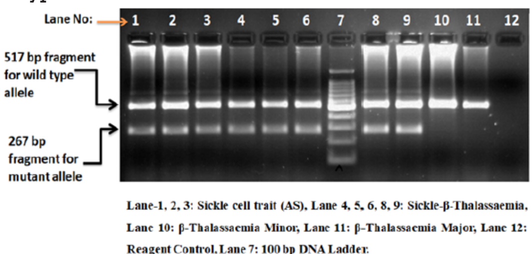


Figure 5: Results of multiplex PCR with sickle cell trait, double heterozygous (Sickle-β-Thalassaemia), β-Thalassaemia minor and β-Thalassaemia major samples.

Whereas in case of double heterozygous *i.e.,* sickle-β-thalassaemia, we got both wild type 517 bp fragment and sickle cell mutant 267 bp fragment leading to interpretation of sickle cell carrier (AS) genotype (as shown in figure 5). So it is obvious that care must be taken in case of thalassaemia and double heterozygous conditions like sickle-β-thalassaemia.

DISCUSSION:

Various methods of high quality such as capillary electrophoresis, cation-exchange high performance liquid chromatography (CE-HPLC) are in recent practice for diagnosis of hemoglobinopathies (Clark & Thein, 2004). Despite PCR based methods, several other similar approaches like restriction enzyme digestion, denaturing gel electrophoresis, allele specific amplification, and the ligase chain reaction method are also available for the characterization of mutations in the β-globin gene. In developing countries where these advanced techniques are not available or limited, electrophoresis and PCR studies are still performed in many laboratories for initial screening (Sheikh *et al.,* 2017). In most patients with sickle cell anemia and other related genetic disorders, the breakpoints are clustered in a very small section of the chromosome and the PCR technique deals with the small amount as well as a small section of DNA. Thus it can be used for the detection in most of the cases with high accuracy. Therefore, a simple method was established for this experiment using PCR technique and gel electrophoresis analysis so that detection of HbS gene might not be considered impossible or expensive.

In this experiment, extraction of DNA from venus whole blood using phenol-chloroform method and detection of β hemoglobin gene and sickle cell disorder using PCR analysis by gel electrophoresis were done. In recent times, DNA extraction is usually done using different sophisticated DNA extraction kits. But in this experiment, all the buffers and solutions were prepared in the laboratory and DNA extraction was done manually following phenol-chloroform method. This method might be time consuming but more cost-effective and DNA can be extracted more accurately than extracting the DNA using the DNA extraction kit.

The results of Hb electrophoresis and PCR were identical for both sickle cell trait (AS) and the healthy controls (AA). This indicates that allele specific PCR can be used for screening of carriers. Despite the availability of SCD screening methods such as the solubility, sickling and peripheral blood film methods and their reliability in the demonstration of patients with SS, they showed variability in their ability to detect the carrier state of sickle haemoglobin (AS). While Hb electrophoresis method can not be used for children <6 months of age in which predominant Hb is HbF, the PCR could be used in both situations. The increased sensitivity provided by PCR analysis has both current and potential applications for prenatal diagnosis, antenatal diagnosis of SCD and confirmation of genotype in neonatal screening at age less than 3 months when HbS is not yet in peripheral blood. This makes the technique more suitable for neonatal screening which ensures a better diagnosis and prognosis in presence of suitable medical care before the clinical onset of the disease.

Our findings were further supported by many studies conducted to evaluate molecular methods applied for screening and diagnosis of SCD. Martinez *et al.,* conducted a study in Venezuela dealing with the application of the PCR for the diagnosis of sickle cell anemia and reported that PCR is one of the fundamental technical bases for establishing a newborn screening program.

Yue *et al.,* evaluated PCR- high resolution melting (HRM) analysis as a rapid tool for screening of SCD; he concluded that HRM is a simple, high efficiency approach for screening of SCD and particularly suitable application in the African area. Ayatollah *et al.,* conducted study for molecular analysis of Iranian families with SCD; he reported that PCR- restriction fragment length polymorphism (RFLP) is a simple, sensitive, and rapid technique, and also has application that is important for the prenatal diagnosis of SCD.

The knowledge of the molecular pathology of hemoglobinopathies has been applied to molecular diagnosis, carrier identification, and neonatal disease diagnosis and has resulted in a decrease in the incidence of the homozygous state in several at-risk populations.

CONCLUSION:

PCR for detecting SNPs like SCA has the potential to offer rapid and inexpensive diagnosis. The SNP genotyping using single tube multiplex PCR system described here is a potential tool for the rapid, easy and cost effective genotypic detection of normal healthy individuals, sickle cell trait and sickle cell anemia patients. This system can also be applied as a screening tool for diagnosis of sickle cell anemia with cord blood samples, samples from neonates & children of < 6 months of age, and persons having a recent history of blood transfusion. Care must be taken with double heterozygous samples like sickle β-thalassaemia because thalassaemia mutation will not be detected by this system.

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