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DIAGNOSIS OF SICKLE CELL ANEMIA BY Dde 1 ENZYME BASED RFLP

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ABSTRACT
Sickle cell anemia is an autosomal recessive genetic disease, resulted by the substitution of valine for glutamic acid at position 6 of the beta globin gene leads to configurational change in hemoglobin of RBC and finally alteration in shape (sickle cell) and loss of retention capacity of oxygen. Its inheritance is based on Mendelian pattern and abundantly found across the world. Its diagnostic confirmation is always a confusing problem for the health worker. In the present study a molecular method for the confirmed diagnosis has been worked out by RFLP method using Dde1 enzyme. After DNA isolation, PCR amplification and RFLP application by Dde1 enzyme, one new bands of treated DNA with 376 bp and diagnostic band for sickling besides normal bands of 201bp and 175bp was found. The new bands of 376bp by using Dde1 enzyme confirm sickle cell anemia.

INTRODUCTION

As a global health problem, sickle cell anemia affects many world populations. Since its initial description in many country almost a century ago, scientists and medical researchers have continued to better understand the pathophysiology of this inherited disease, while simultaneously attempting to find more effective therapies and ultimately a cure. Sickle cell disease refers to a collection of blood disorders characterized by a hemoglobin variant called (HbS).

A single base change (A to T) in the β-globin chain causes the substitution of amino acid glutamic acid to valine, leads to sickle cell anemia. Hemoglobin S. is freely soluble when fully oxygenated, under conditions of low oxygen tension. The red cells become grossly abnormal assuming a sickle shape leading to aggregation and hemolysis. People with sickle cell anemia inherit two copies of sickle cell gene, where as people with one copy of sickle cell gene leads to sickle cell trait. The β -globin gene (HB) is located on the short arm of chromosome 11. The HBB gene is located in region 15.5 on the short (p) arm of human chromosome 11 (11p15.5).The normal allelic variant for this gene is 1600 base pairs (bp) long and contains three exons. The intron-free mRNA transcript for the HBB gene is 626 base pairs long.

The first case report in U.S. literatures occurred in 1910, when Herrick a cardiologist described the physical attributes of the disease as peculiar, elongated and sickle shaped erythrocytes causing distortion of red blood cells by the aggregation of abnormal hemoglobin molecules. Neel (1949) and Beet (1949) independently reported that the mode of inheritance was a recessive gene. Ingram (1957) described the molecular abnormality to a substitution of valine, an aminoacid for glutamic acid at the β - 6 position of the globin chain. Pauling *et al.* (1949) demonstrated the electrophoretic abnormality in sickle hemoglobin (hbs), which migrated differently than normal hemoglobin in an electric field.

Historically, the disease had been known for generations in Africa. The highest prevalence of HbS is in tropical Africa and among blacks in countries that participated in the slave trade. It occurs with lower frequency in the Mediterranean basin, Saudi Arabia, and parts of India. (Pagnier *et al.,* 1984). In India maximum numbers of homozygous sicklers are found in the states of Madhya Pradesh and Chhattisgarh followed by Gujrat, Maharashtra and Andhra Pradesh. Lehman and Cutbush (1952) first presented a report on geographical distribution of sickle disorder in India. Estimates indicate that the sickle cell trait or the carrier state occurs in 10-30% of many, predominantly tribal, populations throughout central India and various studies suggest that there may be between 500,000 and 1,000,000 cases of the disease in India. (Bunn, 1997; Balgir and Sharma, 1988; Bhatia and Rao, 1988)

The pathology of SCD is complicated, in addition to moderate to severe anemia it often includes infarction and resorption of the spleen during childhood, which results in diminished immune function. Growth and development are delayed. Bone and eye disorder may occur. Vaso-occulusive crises punctuate the causes of the disease in many patients and they may be characterized by unbearable pain in the legs and arms, back, chest, or abdomen. Also affected systems are cardiovascular, pulmonary and renal systems.

Restriction Length Fragment Polymorphisms, (RFLP's), are DNA differences that are inherited and can be used as genetic markers for diseases such as sickle cell anemia. In this study RFLPs are used to find out the point mutation in beta globin gene. RFLPs arise because mutations can create or destroy the sites recognized by specific restriction enzymes, leading to variations between individuals in the length of restriction fragments produced from identical regions of the genome. Restriction enzymes are used for analysis such as Dde1, MstII, MsII, etc. Our study includes Dde1 restriction enzyme for RFLPs analysis in sickle cell anemia patient.

MATERIALS AND METHODS

Total 30 samples of blood were collected from Pt. J.N.M Medical College, Raipur, Chhattisgarh, India with kind permission of donor following institutional ethical committee. Blood was collected by glass syringe in EDTA for detection of sickle cell anemia. First solubility test was done followed by cellulose acetate electrophoresis.

After electrophoresis study, DNA was isolated from each sample following method (Thangangraj *et al.,* 2002) and the bands were observed by agarose gel electrophoresis. DNA sample was amplified by PCR with reaction mixture NFW-16.0 μL, agile buffer b-2.5 μL, dNTPs – 2.5 μL, Primer F - 1 μL, Primer R- 1 μL, Agile Taq polymerase- 1 μL, DNA- 1 μL. Reverse primer 5´GAGTGCACAGATCCCCAAAGGACTCAAA GA3´, and forward primer 5´ACCTCACCTGTGGAGCC AC3´(Fig.1, 2). The programme of amplification was denaturation -94ºC for 1 minute, annealing -56ºC for 1 minute 30 seconds extension - 72ºC, it was repeated for 35 times; final extension at 72ºC for 7 minutes cooling at 4ºC.After amplification the amplified DNA was analyzed in 1% Agarose gel. After gel extraction restriction digestion was performed by the help of Dde1 enzyme for that the mixture was prepared with DDW-7.5 μl, NEB buffer 3 (10x) 1.5 μL, enzyme- 1.0 μL, PCR product 5.0 μL; incubated overnight at 37ºC. The product was analysed in 1.5% agarose gel (Fig. 3).

RESULTS AND DISCUSSION

Out of 30 samples by solubility test 20 were found positive and by electrophoresis 10 samples were found homozygous (HbSS) and 10 heterozygous (HbAS). Restriction fragment length polymorphism analysis by the help of Dde1 enzyme (isolated from Desulfovibrio desulfuricans) showed three types of banding pattern. The normal sample has showed two bands of 201bp and 175bp, homozygous sample has showed bands of 376bp and heterozygous sample showed all three bands of 201bp, 175bp with 376bp.

In the present finding the polymorphism in restriction endonuclease digestion pattern of human DNA fragment that contains beta globin structural gene was detected. The Dde1 enzyme recognized the DNA sequence of CTNAG where A is substituted by T in sickle cell anemia due to point mutation. The Dde1 site got affected in sickle cell anemia due to mutation so enzyme failed to recognize the site resulting a new fragment of 376bp. Previously some authors have also used various restriction enzyme for detection of sickle cell anemia viz. MSTII, MSII etc. Nagel *et al.* (1985) have expressed the ability to differentiate milder form of sickle cell disease by means of molecular haplotype analysis and their significant use in prenatal diagnosis.

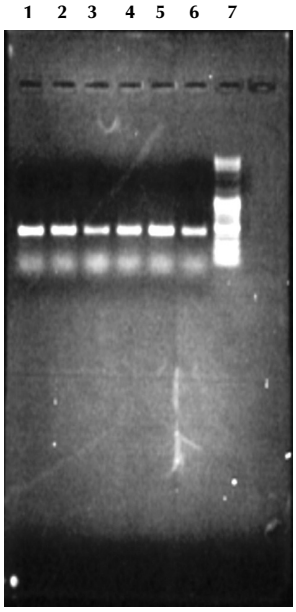

Figure 1: Gel picture showing the PCR amplified product (444 bp) of specific β-globin gene
Lane 1-6: different DNA samples of sickle cell anemia; Lane 7 : 100-bp DNA ladder (medox)

Indirect diagnosis by means of linkage analysis with RFLPS is limited by the need for a sufficient number of informative relatives for pedigree analysis and the potential for recombination within the gene locus. The DNA polymorphism identified in the β -globin gene complex are in sufficiently close linkage to reduce this risk; however, a recombination hot spot located 5´ to the beta gene could allow a crossover in every 300-400 meiosis and could result in an error in one in every 175 cases.(Chakravarti *et al.,* 1984). However, previously reported studies of the use of DNA polymorphism for prenatal diagnosis of β -thalassemia have been highly accurate despite risk (Boehm *et al.,* 1983)

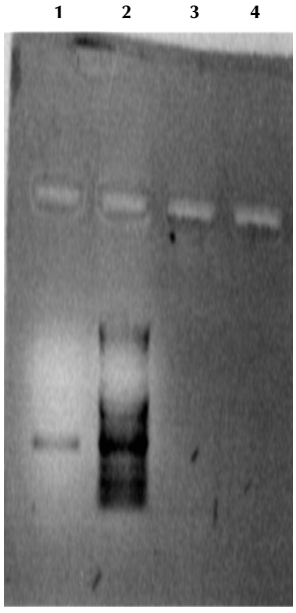

Figure 2: Gel extracted product of PCR amplified DNA (444-bp)
Lane 1: Gel extracted product; Lane 2: 100-bp DNA ladder (medox) (Inverted image)

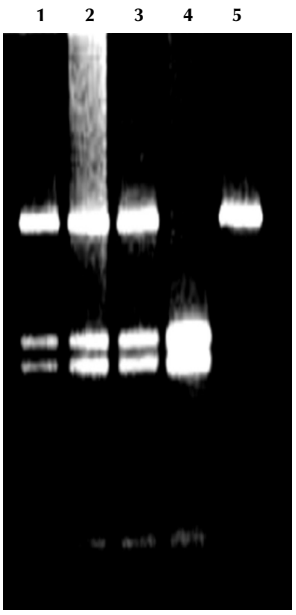

Figure 3: Gel picture showing *Dde*-I- digested PCR product
Lane 1-3: AS (Size 376-bp, 201-bp and 175-bp); Lane 4: AA (Size 201-bp, 175-bp); Lane 5: SS (Size 376-bp)

Recombinant DNA technology provides the methodology for prenatal diagnoses of an every increasing number of genetic disorders as more genes are isolated. Optimization of molecular diagnostic techniques has been demonstrated through the use of both miniaturized blot-hybridisaton techniques, which increase the sensitivity of detection to 50-100ng of DNA, and mutation specific analyses using restriction endonucleases or synthetic oligonucleotide probes (Conner et al. 1983; Orkin *et al.,* 1983; Law *et al.,* 1984).

In the present study mutation specific restriction endonucleases was successfully used for detection of sickle cell anemia. This method will be useful for mass screening programme for this hemoglobinopathies.

Driscoll *et al*. (1987) have reported an evaluation of 55 pregnancies at risk for a sickle hemoglobinopathy prenataly diagnosed by restriction endonuclease analysis, with endonucleases MstII and HpaI, of aminocyte DNA. In this report aminocytes were the source of DNA and 42 cases at risk for SS were analysed with MstII, which cleaves at the sequence CCTNAGG occurring at codons 5, 6, and 7 of the normal β^A gene yield a 1.15-Kb DNA fragment. The sickle cell mutation results in loss of the MstII recognition site and cleavage at an MstII site further 3′, to give a 1.35-Kb sickle cell fragment. In 35 of 42 cases at risk for SS, DNA from both parents was analyzed for MstII heterozygosity at the time of fetal diagnosis. MstII was specific for the β^S gene in all parents and siblings studied. All cases at risk for SC were analysed with MstII and HpaI. MstII will exclude the diagnosis of SC in 50% of cases in which there is a single 1.15-Kb fragment. The presence of two DNA fragments is compatible with either AS or SC. The 13-Kb HpaI fragment, however, is in linkage disequilibrium with the beta c gene in >95% of cases. Extended pedigree analysis was carried out in nine of the 11 cases. The use of MstII excluded the diagnosis in five of the 11 cases, that is, a single 1.15-Kb fragment was observed to be predictive of either an AA- or AC- unaffected fetus. Diagnosis of the remaining six cases required the use of the HpaI polymorphism to differentiate SC from AS genotypes. Two cases at risk for both SS and S β-thalassemia were also evaluated with MstII and HpaI after pedigree analysis showed HpaI to be informative. One fetus had a single 1.35-Kb fragment compatible with SS, a condition substantiated by linkage analysis with HpaI. This fetus was terminated, and a fetal blood sample confirmed homozygous SS. The second fetus had two MstII fragments, 1.14-Kb and 1.35-Kb, that could be compatible with either AS or S β-thalassemia. HpaI analysis predicted S β-thalassemia, which was confirmed at birth.

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