INTRODUCTION

As a global health problem, sickle cell anemia affects many world populations. Since its initial description in many countries 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 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 band 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.

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 band 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.

KEY WORDS
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Restriction Length Fragment Polymorphisms (RFLPs) 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´GAGTGCCAGATCCCCAAAGGACTCAAA GA3´, and forward primer 5´ACCTCACCTGTGGACCC 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 analyzed 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, MstII 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.

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).
MstII was specific for the alleles at MstII heterozygosity at the time of fetal diagnosis. In 35 of 42 cases at risk for SS, DNA from both parents was analysed for MstII heterozygosity at an MstII site further 3′, to give a 1.35-Kb sickle cell fragment. This mutation results in loss of the MstII recognition site and cleavage of normal sequences CCTNAGG occurring at codons 5, 6, and 7 of the β gene. The presence of a 1.35-Kb DNA fragment is diagnostic of SS. The sickle cell hemoglobin genotype SS from AS genotypes. Two cases at risk for both SS and S-β-thalassemia were also evaluated with MstII and Hpal after pedigree analysis showed Hpal to be informative. One fetus had a single 1.35-Kb fragment compatible with SS, a condition substantiated by linkage analysis with Hpal. This fetus was excluded at birth. 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. Hpal analysis predicted S-β-thalassemia, which was confirmed at birth.

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