# MOLECULAR ANALYSIS OF SICKLE CELL ANEMIA IN SOUTH INDIAN PATIENTS


**SUBMITTED BY**

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**DEPARTMENT OF GENETIC ENGINEERING**




**BHARATH INSTITUTE OF HIGHER EDUCATION AND RESEARCH**

# MOLECULAR ANALYSIS OF SICKLE CELL ANEMIA IN SOUTH INDIAN PATIENTS

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**PRUDHVI RAJ (U17BR060)**

**In Partial Fulfillment For The Requirement**

**Of**

**The Degree Of Bachelor Of Science**

**In**

**GENETIC ENGINEERING**

**Under The Guidance Of**

**Mrs. VIJAYALAKSHMI (Asst Prof)**

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# CERTIFICATE

This is to certify that the project work reported in the dissertation entitled **"Molecular Analysis Of Sickle Cell Anemia In South Indian Patients"** is carried out by **R. PRUDHVI RAJ** under the supervision of **Dr. SYEDA ZUBEDA** Genome Foundation, Hyderabad.

As, declared by the candidate the work is original and has not been submitted in part for any other degree.

Head of Department

# DECLARATION

I declare that this dissertation work entitled **"MOLECULAR ANALYSIS OF SICKLE CELL ANEMIA IN SOUTH INDIAN PATIENTS"** is a record of research work which is original, and carried out by me from November 2020 to February 2021, under the guidance of **Dr. SYEDA ZUBEDA,** Scientist Genome Foundation, Hyderabad.

**Date: -**

**Place: -**

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| SNO | SHORT FORM | ABBREVIATIONS |
|---|---|---|
| **1** | **HBS** | **Sickle Hemoglobin** |
| **2** | **DNA** | **Deoxyribonucleic acid** |
| **3** | **SDS** | **Sodium dodecyl sulfate** |
| **4** | **NaCl** | **Sodium chloride** |
| **5** | **KCl** | **Potassium chloride** |
| **6** | **Mgcl2** | **Magnesium chloride** |
| **7** | **EDTA** | **Ethylenediamine tetra acetic acid** |
| **8** | **DMSO** | **Dimethyl sulfoxide age** |
| **9** | **AGE** | **Agarose gel electrophoresis** |
| **10** | **ETBR** | **Ethidium bromide** |
| **11** | **TAE** | **Tris acetate EDTA** |
| **12** | **PCR** | **Polymerase chain reaction** |

| | | |
|---|---|---|
| **13** | **dNTP** | **Deoxynucleotide triphosphate** |
| **14** | **ddNTP** | **diDeoxynucleotide triphosphate** |

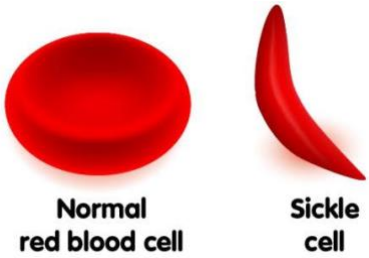
# ABSTRACT

Sickle cell disease (SCD) is a very devastating condition caused by an autosomal recessive inherited haemoglobinopathy. This disease affects millions of peoples globally which results in serious complications due to vasoocclusive phenomenon and haemolysis. This genetic abnormality is due to substitution of amino acid valine for the glutamic acid at the sixth position of beta chain of haemoglobin. This disease was described about one hundred year ago. The haemoglobin S (hbS) produced as result of this defect is poorly soluble and polymerized when deoxygenated. Symptoms of sickle cell disease are due to chronic anaemia, pain full crises, acute chest syndrome, stroke and susceptibility to bacterial infection. In recent years measures like prenatal screening, better medical care, parent education, immunization and penicillin prophylaxis have successfully reduced morbidity and mortality and have increased tremendously life expectancy of affected individuals. Three principal current therapeutics modalities available for childhood SCD are blood transfusion, Hydroxy urea and bone marrow transplantation. Genetic counseling, continued medical education for health professionals about sickle cell disease, its complications and management is necessary. WHO has actively promoted several national screening programs with dual goals of informing reproductive choice and thereby reducing the number of severely affected children.

# INTRODUCTION

## SICKLE CELL ANEMIA

Sickle cell anemia is an inherited (genetic) condition in which, it effects red blood cells to change into sickle shape, which produce episodes of pain and other symptoms like lack of fluid in the body and low oxygen levels. Some of the long term complications are pain in the joints, breathing problems etc. Diagnosis is done by testing the blood in newly born babies and also in adults . Sickle cell disease is different from sickle cell trait, sickle cell trait is a carrier of sickle cell gene it is normally asymptomatic [(1)].




(Figure1)[2] Normal red blood cell changes to sickle cell

## INCIDENCE

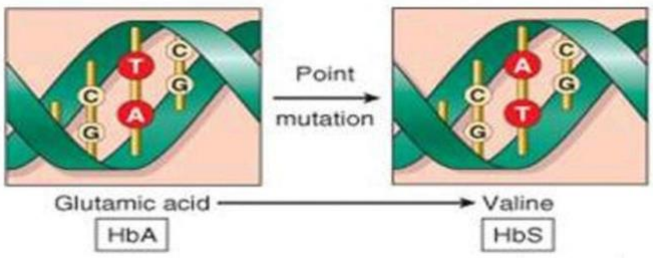
Sickle cell disease (SCD) affects millions of people throughout the world and is particularly common among those whose ancestors came from sub-Saharan Africa; Spanish-speaking regions in the Western Hemisphere (South America, the

Caribbean, and Central America); Saudi Arabia; India; and Mediterranean countries such as Turkey, Greece, and Italy[3.]

India has the largest concentration of tribal populations globally. They are believed to be the early settlers in the country and are considered to be the original inhabitants. According to the Census of India 2020 [4] , Based on these surveys, prevalence of sickle gene is found to be 0-18% in north eastern India, 0-33.5% in western India, 22.5-44.4% in central India and 1-40% in southern India and the gene frequency of Hb-S varies between 0.031- 0.41.The States of Madhya Pradesh, Maharashtra, Odisha, Gujarat, Rajasthan, Jharkhand, Chhattisgarh, Andhra Pradesh, West Bengal and Karnataka account for around 83 per cent of the total scheduled tribe population in the country and majority of these tribal groups live in rural areas. In all, 461 scheduled tribes have been listed [5] and they have their own characteristic cultural patterns, languages and social systems, by and large keeping to themselves. However,[6] concluded that "several thousand years ago, the entire subcontinent underwent a period of massive intermarriage, shuffling its population′s genetic deck so thoroughly that it left clear traces even in the genomes of today′s most isolated tribes".

## GENETICS AND INHERITANCE

Sickle cell disease is a hereditary hemoglobinopathy resulting from Inheritance of a mutant version of the β-globin gene (βA) on Chromosome 11, this gene codes for

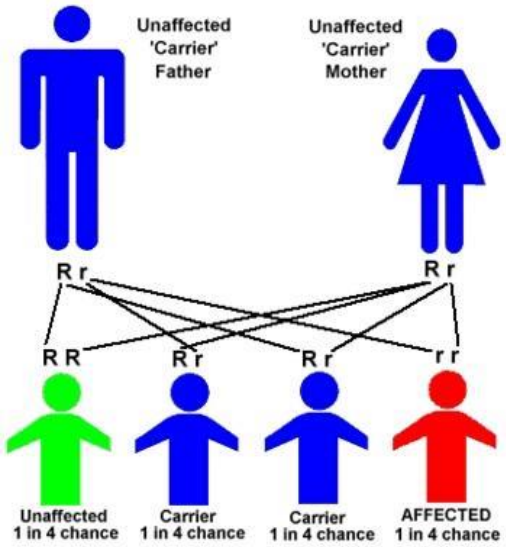


assembly of the β-globin chains of the protein hemoglobin A. The mutant β-allele (βS) codes for the production of the variant hemoglobin, hemoglobin S. The heterozygous carrier state is known as sickle cell trait (SCT)[7]. The sickle cell gene mutation is a point mutation in the sixth codon of exon 1 in the βA gene, replacing adenine with thymine (guanine-adenine-guanine---guanine-thymine-guanine)[8]

(Figure 2) [9] adenine is substituting with thymine

Homozygosity for the sickle mutation (i.e., HbSS disease) is Responsible for the most common and most severe variant of SCD. Several other genetic variants of SCD result from the interaction of Different mutations of the human b-globin genes[10] . Sickle-cell disease symbolizes all genotypes containing at least one Sickle gene, in which HbS makes up at least half the haemoglobin Present.

Inheritance of sickle cell disease refers to a collection of autosomal recessive genetic disorders characterized by the HbS gene. Person who is effected with sickle cell anemia have both the copies of variant (HbSS). Some of the individuals who are effected with two different variants of sickle cell disease is known as compound heterozygous, They contain one copy of the HbS and another copy of HbC or HbP. These individuals produce mixture of hemoglobins. Carrier individuals one copy of sickle variant and another copy of normal hemoglobin. The carriers are known to have sickle cell trait in which they are in mild condition but some studies found that sickle cell trait have a risk of sudden death during physical activities[11].



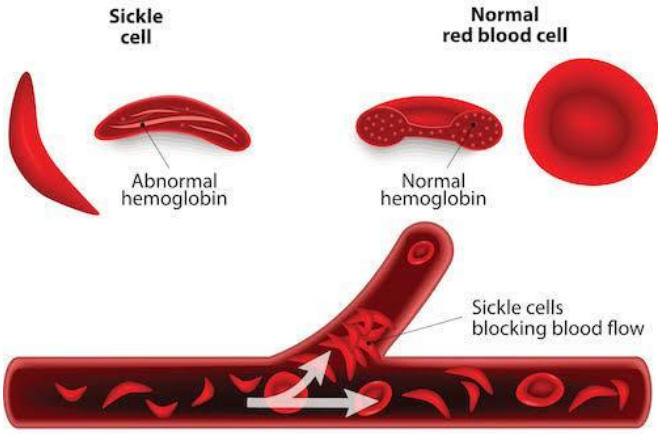

( Figure 3)[12] genetic inheritance of sickle cell

Haemoglobin is affected by the sickle cell genes. Haemoglobin is present in the red blood cells which carries oxygen and gives blood its red color. HbA gene is present in the normal hemoglobin sickle cell genes make the normal genes to HbS. HbS gene makes the red blood cells shape from doughnut to sickle shape cells this process is called sickling. Complications of sickling are infection, dehydration, low oxygen levels etc.

HbS gene present in the sickle cells are harder and less flexible than normal red blood cells. This type of cells get stuck in the blood vessels and block them. These

blockage causes sickle cell crisis. Sickle cells are easily destroyed than normal red blood cells which causes lack of red blood cells (anaemia).

(Figure 4) [13] sickle cell blockage




## CAUSE OF SICKLE CELL DISEASE:

Sickle cell genes, which are transferred one from each parent. When a person having only one allele it is called sickle cell trait which is asymptomatic and mild. Mostly SCD is where you have both the alleles called sickle cell anemia. Medical form of this gene is HbSS.

**HbS gene:** HbS gene arises from a mutation substituting thymine for adenine in the sixth codon of the beta chain gene, **GAG** to **GTG** this causes codon of valine instead of glutamate in position 6 of the Hb beta chain. The resulting hb has the physical properties of forming polymers under deoxy condition[14].

## SYMPTOMS OF SICKLE CELL DISEASE (SCD):

Symptoms of sickle cell disease are not frequent. There are episodes of symptoms, but in between episodes the person will be well and normal. The episodes of symptoms are because the red blood cells are normal most of the time but something

makes cell to much sickle then the symptoms appear due to the sickling know as sickle cell crisis. Some people have frequent symptoms but some are have very few in which sickle cell is unnoticeable. Most of the people with sickle cell disease are mild and unnoticeable.[15]

The various symptoms that can occur if you have SCD include:

**EPISODES OF PAIN:**

This is also called a pain crisis or a vascular occlusion crisis. It occurs when sickle-shaped red blood cells block small blood vessels in the bone, causing pain. Pain usually occurs in bones and joints. The pain can be mild or severe, or it can come on suddenly. Common symptoms in infants and toddlers are swollen and painful bones in the fingers and toes. This is called dactylitis.[15]

**INFECTIONS:**

People with SCD are especially susceptible to severe infections with certain types of bacteria (bacteria) that can cause pneumonia, meningitis, sepsis, or bone infection. These include pneumococcal bacteria, Haemophilus influenzae type B, meningococcal and salmonella bacteria, which can infect bones. Symptoms of infection include fever, general discomfort, and pain in the affected area of the body. Children with SCD are at increased risk of serious or life-threatening infections. It is important to see a doctor immediately if you suspect an infection or feel unwell.[15]

**ACUTE CHEST SYNDROME:**

It happens when the blood vessels in the lungs are blocked and can sometimes be caused by a lung infection. Symptoms include chest pain, fever, and shortness of breath. Babies and toddlers have less noticeable symptoms and usually feel unwell,

lack energy (sleepiness), are anxious, or breathe quickly. Acute breast syndrome is very serious and requires immediate hospitalization if suspected. Acute breast syndrome can begin a few days after a painful sickle crisis. This occurs most often in women who are pregnant or have recently given birth.[15]

**ANAEMIA EPISODES:**

Anemia is a lack of hemoglobin in the blood. As mentioned above, people with SCD usually have mild anemia that does not cause problems. However, sometimes people with SCD can develop severe anemia, which can be serious. It can appear very suddenly or gradually. Urgent care may be required.

1. Fatigue, fainting, shortness of breath, dizziness, nausea (nausea), or rapid breathing, aggravated by exercise.
2. Infants and toddlers may be lethargic, eat little, or generally feel unwell.
3. Pale skin color (best seen on lips, tongue, nails, or eyelids).
4. In children, the spleen enlarges rapidly, causing sudden and severe anemia. Hypertrophy. The hypertrophy is on the abdomen and is felt. Parents can show their children how the spleen feels. If the spleen is growing rapidly, this is a sign that immediate treatment is needed.[15]

## DIAGNOSIS

The diagnosis is made by a blood test. The blood sample is analyzed to see what type of haemoglobin is present in the blood (using a test called haemoglobin electrophoresis or other methods).

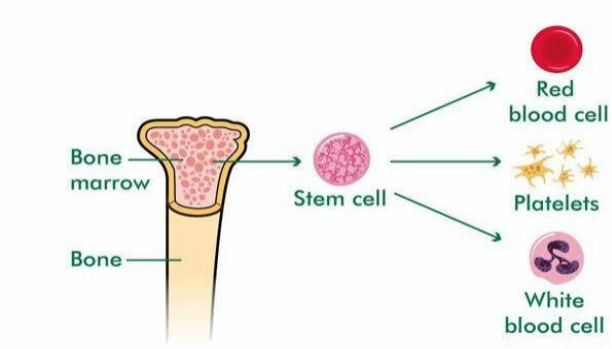
## TREATMENT FOR SICKLE CELL DISEASE:

Sickle cell anemia cannot be cured but there are some form of treatment to control the sickling and infections

## PRINCIPLES OF TREATMENT

1. Specialist doctors or team experienced in treating patients with SCD are helpful.
2. Symptoms of sickle cell are sudden so you should be ready to see doctor near you and get treatment urgently
3. Treatment should be tailored to your individual needs
4. Preventive treatments against infection should be taken and frequent checkups needed

   Stem cell transplant is the only available treatment that can cure SCD. It is only used for severe SCD. Its use is limited by side-effects of the procedure and the availability of suitable donors.[15]




(figure 5)[16] stem cell transfusion

## STAYING HEALTHY :

1. A daily antibiotic is usually recommended (penicillin, or erythromycin if you are allergic to penicillin). This is especially important to protect against serious infections in children aged under 5 years.
2. Immunizations: all the usual childhood vaccinations are advised, PLUS you should have vaccinations against meningitis and hepatitis B, PLUS an influenza (flu) vaccination once a year. These vaccines are recommended both for adults with SCD and for children with SCD.
3. Vitamin supplements: extra folic acid is usually recommended. This helps the body to make new red blood cells.
4. Travel: if you go to a country where there is malaria, be extra careful to take malaria prevention medication and to prevent mosquito bites (people with SCD can get very ill from malaria).
5. Avoid smoking (which is bad for blood vessels) and excess alcohol.

Factors which can trigger sickling include:

1. Cold.
2. Lack of oxygen.
3. Lack of fluid in the body (dehydration).
4. Hard exercise.
5. High temperature (fever).
6. Infection.

## TREATMENT OF SICKLING EPISODES:

Treatment usually involves:

1. Painkillers. Depending on the amount of pain, you can take various types of pain medication. Mild painkillers are paracetamol or ibuprofen. Moderate ones are codeine or dihydrocodeine. A strong painkiller such as morphine may be needed for severe pain - this is usually given in hospital.
2. Good hydration. This usually means drinking extra fluid, or sometimes a drip into one of your veins, which is needed if you are more unwell or cannot drink.
3. Oxygen. This is usually given to you through a face mask in hospital. If you are not getting enough oxygen then more of your red cells may become sickle-shaped.
4. Antibiotics. These are used if you have an infection, or when infection is suspected.

**BLOOD TRANSFUSION:**

Blood transfusion is a useful treatment for some situations, such as acute chest syndrome or severe anaemia. It can also be used to help prevent or treat certain complications. The transfusion helps because it adds normal red blood cells to the blood. This corrects anaemia and reduces the effects of sickling. There are potential side effects from blood transfusions. Therefore, transfusions are given for a specific need, rather than routinely.[15]

(figure 6) [17] blood transfusion

## COMPLICATIONS OF SICKLE CELL DISEASE (SCD) AND HOW THEY ARE PREVENTED AND TREATED :

### POSSIBLE COMPLICATIONS IN CHILDREN GROWTH, DEVELOPMENT AND NUTRITION –

As with any long-term illness, a child with SCD may grow more slowly than usual, or be undernourished if the illness affects their appetite. Your child's growth, development and nutrition should be checked regularly, and nutritional supplements may be given if needed. Some children with SCD take longer than usual to gain control of their bladder at night, so may wet the bed (nocturnal enuresis). Various treatments can help. For teenagers, puberty may start about 2-3 years later than average. The growth of bones can also be affected.

For example, there may be changes in the hip or shoulder joints due to blocked blood vessels in that part of the bone. If a joint is severely affected, surgery may be needed.

**STROKE OR BRAIN INJURY :**

This is a serious complication and affects about 1 in 10 children or teenagers with SCD. If sickle cells block blood vessels in the brain, this may cause a stroke. There may be symptoms of stroke such as weakness of the face or limb, or speech difficulty. For some children, there may be no obvious symptoms. However, many tiny strokes may cause a subtle brain injury and make learning more difficult.

Strokes are treated with blood transfusion, which improves blood flow to the brain. Also, research has found that regular blood transfusions help to prevent strokes. An ultrasound test called a transcranial Doppler can be used to look at the blood flow to the brain. This helps doctors to decide whether your child needs blood transfusions for prevention. Children aged 3 years should be offered these scans.

**SPLEEN PROBLEMS:**

The spleen is an organ located in the tummy (abdomen), in the top left-hand side. Its function is to help the immune system. Sickle cells can block blood vessels in the spleen. This can make the spleen swell up suddenly with blood - in effect, it is like losing blood into the spleen. This is one cause of sudden and severe anaemia, when your child becomes suddenly ill. The medical term is splenic sequestration. It needs urgent treatment with a blood transfusion.If this problem happens more than once then one option is surgery to remove the spleen. However, by adulthood the problem normally resolves because the spleen becomes hard and cannot swell.

**LUNGS, HEART AND KIDNEYS:**

Any of these organs may suffer some damage. Therefore, you will normally be offered regular checks on your heart, lungs and kidney function. Various treatments can help.

**AIM:** To screen high risk individuals in South Indian population for sickle cell anemia. Which includes,

1. Isolation of DNA
2. Quantification of DNA by qubit fluorometer
3. Polymerase chain reaction (PCR)
4. Agarose gel electrophoresis
5. Sequencing of the sample by genetic analyzer which include;

6. Exosap cleanup
7. Cycle sequencing
8. Post PCR cleanup
9. Loading the sample in genetic analyzer
10. Collecting the data
11. Collected data is analyzed in codon code aligner

# REVIEW OF LITERATURE

12. The tribal communities constitute a major part of India. Sickle cell disease is a genetically transmitted hemo-Globinopathy. Sickle hemoglobin was first detected by Lehman and Cutbush in 1952 among the tribals from Nilgiris. During the Last 54 years, several groups of investigators conducted hospital based epidemiological surveys in various ethnic groups. Based On these surveys, prevalence of sickle gene is found to be 0-18% in north eastern India, 0-33.5% in western India, 22.5-44.4% in Central India and 1-40% in southern India and the gene frequency of Hb-S varies between 0.031- 0.41. Wide variability in the Prevalence of Hb-S trait is observed in population groups within small geographical areas. It causes high degree of morbidity and Mortality. There is paucity of data with respect to its prevalence, due to geographical and social barriers, also because relatively few primary and specialty care health facilities exist in tribal areas, and gaping disparities in

health status of tribal, as compared to those in Metropolitan areas, are evident. With a large population, increase rapid birth rate, and consanguineous marriage practices, there is a dangerously high prevalence of genetic disorders among tribal populations. Epidemiological studies confirmed that sickle cell Anemia is rampant in the tribal populace, the prevalence of homozygotes for the sickle gene calculated to be over 20% with an Estimated five million individuals predicted as carriers. It is recommended that genetic health services be integrated into existing Primary health care and medical services to combat the epidemic.

Sickle cell disease (SCD) is a monogenetic disorder due to a single base-pair point mutation in the β-globin gene resulting in the substitution of the amino acid valine for glutamic acid in the β-globin chain. Phenotypic variation in the clinical presentation and disease outcome is a characteristic feature of the disorder. Understanding the pathogenesis and pathophysiology of the disorder is central to the choice of therapeutic development and intervention. In this special edition for newborn screening for haemoglobin disorders, it is pertinent to describe the genetic, pathologic and clinical presentation of sickle cell disease as a prelude to the justification for screening. Through a systematic review of the literature using search terms relating to SCD up till 2019, we identified relevant descriptive publications for inclusion. The scope of this review is mainly an overview of the clinical features of pain, the cardinal symptom in SCD, which present following the drop in foetal haemoglobin as young as five to six months after birth. The relative impact of haemolysis and small-vessel occlusive pathology remains controversial, a combination of features probably contribute to the different pathologies. We also provide an overview of emerging therapies in SCD.

Sickle cell disease (SCD) is one of the most common genetic causes of illness and death in the world. This is a review of SCD in Africa, which bears the highest burden of disease. The first section provides an introduction to the molecular basis of SCD and the pathophysiological mechanism of selected clinical events. These consecution discusses the epidemiology of the disease(prevalence, morbidity, and mortality), at global level and within Africa. The third section discusses the laboratory diagnosis and management of SCD, emphasizing strategies that been have proven to be effective in areas with limited resources. Through out the review, specific activities that require evidence to guide healthcare in Africa, as well as strategic areas for further research, will be highlighted.

Sickle cell disease (SCD) predominates in sub-Saharan Africa, East Mediterranean areas, Middle East, and India. Nigeria, being the most populous black nation in the world, bears its greatest burden in sub-Saharan Africa. The last few decades have witnessed remarkable scientific progress in the understanding of the complex pathophysiology of the disease. Improved clinical insights have heralded development and establishment of disease modifying interventions such as chronic blood transfusions, hydroxyurea therapy, and haemopoietic stem cell transplantation. Coupled with parallel improvements in general supportive, symptomatic, and preventive measures, current evidence reveals remarkable appreciation in quality of life among affected individuals in developed nations. Currently, in Nigeria and other West African states, treatment and control of SCD are largely suboptimal. Improved knowledge regarding SCD phenotypes and its comprehensive care among Nigerian physicians will enhance quality of care for affected persons. This paper therefore provides a review on the aetiopathogenesis, clinical manifestations, and management of SCD in Nigeria.

Sickle cell anemia is still a significant public health issue in underdeveloped and developing countries. Sickle cell disease is one of the most common inherited diseases in Brazil. It affects mainly the mixed race population. Approximately 1 African-Brazilian child is affected with sickle cell disease for every 37,400 children born alive. Hearing loss has been considered one of the main clinical manifestations, especially in children. However, to date, there are just a hand full of studies in Brazil and the Brazilian state of Bahia has the largest African-descended population, attempting to establish the frequency of this event.

Sickle cell trait is not usually regarded as a disease state because it has complications that are either uncommon or mild. Nevertheless, under unusual circumstances, serious morbidity or mortality can result from complications related to polymerization of deoxy-hemoglobin S. A previous study was earlier conducted to study Sickle cell traits and it revealed that there was enhanced lipid per oxidation along with imbalance in the pro-oxidant and antioxidant status in patients with sickle cell anaemia. Moreover, sickle cell traits present with varied problems including increased urinary tract infection in women, gross hematuria, complications of hyphema, splenic infarction with altitude hypoxia or exercise, life-threatening complications of exercise etc. Renal medullary carcinoma in the young, early onset of end stage renal, as well as disease from autosomal dominant polycystic kidney disease are other well known occurrences in sickle cell traits. In view of the above facts, this article aims to review the literature to analyze the health status in sickle cell traits.

The inheritance of genetic disease depends on ancestry that must be considered when interpreting genetic association studies and can provide

insights when comparing traits in a population. We compared the genetic profiles of African Americans with sickle cell disease to those of Black Africans and Caucasian populations of European descent and found that they are less genetically admixed than other African Americans and have an ancestry similar to Yorubans, Mandenkas and Bantu.

With advances in management, the majority of women in the UK with sickle cell disease now Survive to have children. The high risk of fetal and maternal sequelae in their pregnancies Mandates multidisciplinary management involving an obstetrician, a haematologist, an Anaesthetist and a haemoglobinopathy specialist nurse. Hydroxycarbamide, a new treatment For sickle cell disease, is contraindicated in pregnancy. Exchange transfusion may be Indicated in women with serious obstetric or haematological complications. In those with Sickle cell disease, the entire pregnancy is a high-risk period that warrants close monitoring. It is thus important for every obstetrician to be familiar with the condition.

# MATERIALS AND METHODS

## ISOLATION OF DNA

The first isolation of DNA was done in 1869 by Friedrich Miescher. Currently it is a routine procedure in molecular biology or forensic analyses. For the chemical method, there are many different kits used for extraction.

The first crucial step in performing DNA extraction is the collection and the storage of specimen. Peripheral whole blood (the main resource used to obtain DNA for human genetic research studies) can be obtained using Vacutainer tubes treated with EDTA or Sodium Citrate to prevent clotting. Heparin is not recommended because it can inhibit Polymerase Chain Reaction (PCR) or Restriction Fragment Length Polymorphism (RFLP) analysis. Before the DNA extraction, whole blood samples may be conserved for maximum three days at +4°C, or several years frozen (-20°C or -80°C). Storage time and conservation mode affects the DNA recovery ratio.

Types of DNA extraction:

1. Salting out method
2. Phenol-chloroform method
3. Silica gel method and etc.

## Salting out method

**Materials:**

| Sno | Reagents |
|---|---|
| **1** | TKM1: Tris Hcl, EDTA, Kcl,Mgcl2 |
| **2** | TKM2 :Tris Hcl, EDTA, Kcl, Mgcl2,Nacl |
| **3** | SDS |
| **4** | Triton X |
| **5** | Isopropanol |
| **6** | 70% Ethanol |

Method:

- Take 300µl of isopropanol into each of 1.5ml Microcentrifuge tubes and store it in refrigerator
- Take 1.5ml microcentrifuge tubes add 300µl of blood & add 900µl of TKM1 and 100µl of Triton X & vortex it properly
- Centrifuge the tubes at 8000rpm for 10minutes and repeat the same process for 3 times up-to the pellet appears clearly
    - Figure 7 [18]


(Figure 8) [19] centrifuge machine

**CENTRIFUGE MACHINE**

- Discard the supernatant and dislodge the pellet
- Add 260µl of TKM2 and 40µl of SDS & vortex it vigorously
- Leave the tubes for incubation for 20minutes at room temperature
- After 20min mix the contents in the tubes well and add 120ul
    - Of **Nacl** drop by drop along the walls of the tube

- Mix well and centrifuge at 8000rpm for 10min
- Slowly transfer the supernatant into tubes containing chilled isopropanol
- Centrifuge again at 8000rpm for 10minutes
- Discard the supernatant and add 360ul of 70% ethanol to the tubes
- Centrifuge at 8000rpm for 10minutes
- Discard the supernatant and air dry the tubes over night

## QUANTIFICATION OF DNA


(Figure 9)[20] qubit fluorometer

- Qubit fluorometer is used to find the quantity of DNA present in the sample.
- DNA is of two different ranges
- Broad range
- Narrow range
- Kit normally contains standard, buffer, DMSO we are not going to use the standard here.
- Buffer should be 197ul and DMSO should be 1ul and 2ul DNA

- Total qubit tube contains 200ul.
- Prepare master mix which contains the buffer and DMSO and mix them shortly.
- Take 198ul of master mix which means buffer+DMSO in each tube.
- Add 1ul of sample DNA and incubate for 5 minutes and do the qubit check so that we can see the quantity of DNA present in the sample

- DMSO is light sensitive so make sure that lights are off when using DMSO
- DMSO ( Dimethyl sulphoxide)

# POLYMERASE CHAIN REACTION (PCR)

**Polymerase chain reaction** is a method widely used to rapidly make millions to billions of copies of a specific DNA sample, allowing scientists to take a very small sample of DNA and amplify it to a large enough amount to study in detail.

**Principle of PCR:** Polymerase chain reaction (PCR) is a powerful and widely used technique that has greatly advanced our ability to analyze genes. Genomic DNA present in cells contains many thousands of genes. This makes it difficult to isolate and analyze any individual gene. PCR allows specific DNA sequences, usually corresponding to genes or parts of genes, to be copied from genomic DNA in a simple enzyme reaction. Only requirement is that some of the DNA sequence at either end of the region to be copied is known. DNA corresponding to the sequence of interest is copied or amplified by PCR more than one million fold and becomes the predominant DNA molecule in the

reaction. Sufficient DNA is obtained for detailed analysis or manipulation of the amplified gene[21].

To perform PCR we have to design primers based on the targeted region. Primer is a short nucleic acid sequence that provides starting point for DNA synthesis. The primers required for PCR are:

Forward primer (5' to 3')

Reverse primer (5' to 3')

Forward primer attaches to start codon of the template DNA, reverse primer attaches to stop codon of complementary strand of DNA. 5' end attaches to 3'of each DNA

## ESSENTIAL COMPONENTS OF REACTION (PCR) :

| Sno | Components |
|---|---|
| 1 | DNA sample |
| 2 | Primers |
| 3 | Free nucleotides called dNTPs |
| 4 | Tag Polymerase |
| 5 | Buffer |

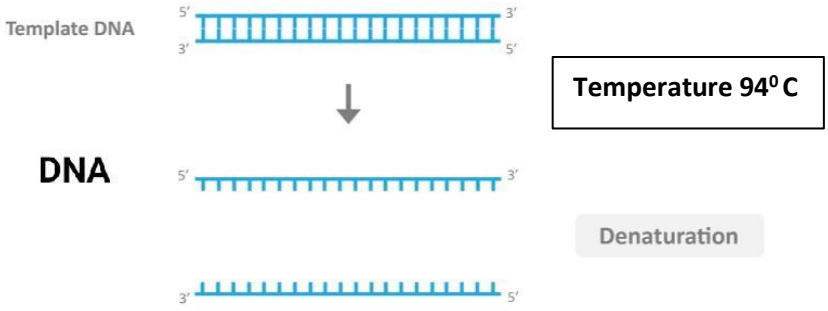
Amplification of the target sequence is achieved by a repetitive series of cycles involving three steps:

- **Denaturation** of the template by heat.

- **Annealing** of the oligonucleotide primers to single stranded target sequences.
- **Extension** of the annealed primers by thermostable DNA polymerase[22].

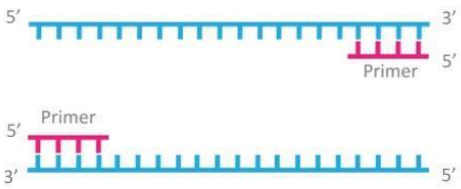
## 1. DENATURATION:

In PCRs catalysed by Taq polymerase, denaturation is carried out at 94-95 ºC, which is the highest temperature the enzyme can withstand for 30 or more cycles without being damaged. During the first cycle, denaturation is carried out for 5 minutes to ensure complete denaturation of the long molecules of template DNA. But at times such longer duration of denaturation may be deleterious. Denaturation for 45 seconds at a temperature of 94-95 ºC is recommended for routine amplification of linear DNA templates containing 55% or lesser amount of G+C. Higher temperatures may be required to denaturate templates containing higher amounts of G+C. In addition, longer the DNA templates, greater is the denaturation time required. If denaturation temperature is too low or if time is too short, only the A–T rich regions of template DNA will




be denatured. Such DNA will re-anneal back when denaturation temperature is reduced later during PCR cycle[23].

## 2. ANNEALING:



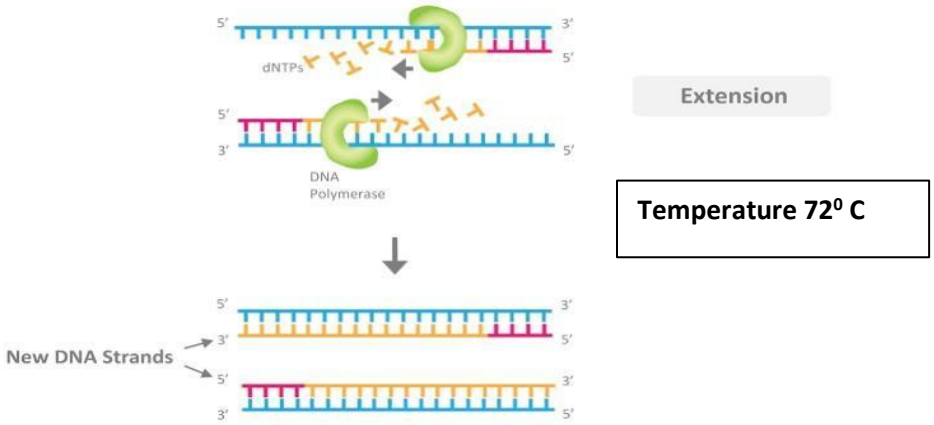

Annealing

Annealing temperatures range from 55-65 ºC depending on the primer sequence and length. Annealing temperature is critical. If annealing temperature is too high, the oligonucleotide primers anneal poorly and the amplified DNA is too low. If annealing temperature is too low, nonspecific annealing of primers may occur, resulting in the amplification of unwanted segments of DNA. Annealing temperature can be optimized by performing series of trial PCRs at room temperatures ranging from 2-100 ºC below the melting temperatures of oligonucleotide primers. Also a sequential series of annealing temperatures can be used in a routine PCR[23].

## 3. EXTENSION :

Extension of from one primer proceeds beyond the sequence complementary to the binding site of the other primer during the first two cycles. In the next cycle, the length of the DNA molecules produced is equal to the segment of DNA delimited by binding sites of primers. From the third cycle onwards,

this segment of DNA is amplified geometrically while the longer amplification products accumulate arithmetically[23].




# AGAROSE GEL ELECTROPHORESIS(AGE)

Agarose gel electrophoresis is a method used in biochemistry and molecular biology to separate DNA or RNA molecules by size. This is achieved by moving negatively charged nucleic acid molecules through agarose matrix with an electric field(electrophoresis). Shorter molecules mover faster and migrate farther than longer ones. DNA gel electrophoresis is usually performed for analytical purposes. Often amplification of DNA via PCR may be used as preparative technique prior to use of other methods such mass spectrometry,RFLP,PCR, cloning DNA sequencing, Sothern blotting for further characterisation.

## Principle:

DNA can be electrophoresed through the gel prepared by melting and re-gelling agarose. Agarose is a co-polymer of D-galactose and 3,6 anhydro L-galactose. It forms a gel by hydrogen bonding and pore size depending on the gel Concentration. The movement of DNA bands within the gel is influenced by agarose concentration. Increasing the agarose concentration of the gel reduces the migration speed and enables separation of smaller DNA molecules. The higher the voltage, the faster the DNA moves but resolution (above about 5-8V/cm).The common dye used make DNA or RNA bands visible for agarose gel electrophoresis is ethidium bromide, usually abbreviated as EtBr.It fluorescence under UV light when intercalated into DNA or RNA. By running DNA through EtBr-treated gel and visualizing it with UV light any band containing more than ~20ng becomes distinctly visible. Since EtBr stained DNA is not visible under normal light, DNA is mixed with negatively charged loading buffers before adding the mixture to the gel. Xylene cyanol and bromophenol are common loading buffers; they run about the same speed as DNA fragments that are 5000bp and 300bp in length respectively, but the precise position varies with the percentage of gel. Most agarose gels are made with between 0.7%(good separation or resolution of large 5-10kb DNA fragments) and 2% (good resolution for small 0.2-1kb DNA fragments) agarose dissolved in electrophoresis buffer. There are number of buffers used for agarose gel electrophoresis. The most common being Tris/acetate EDTA(TAE),Tris/borate EDTA (TBE) and lithium borate(LB).TAE has lower buffering capacity but provides best resolution for larger DNA.

| Sno | Reagents |
|---|---|
| 1 | Agarose gel |
| 2 | TAE buffer ( Tris Hcl, EDTA, Acetic acid) |

| | |
|---|---|
| 3 | EDTA ( Ethidium bromide) |
| 4 | Loading dye |
| 5 | Ladder |

## METHOD:

1. Weight the agarose and add it in the TAE buffer
    - ( 1.5grams in 100ml)
2. Heat the solution in a microwave oven up to agarose is melted clearly
3. Cool down the buffer up-to it reaches 40degrees
4. Add ETBR to the solution and mix it well
5. Take the gel plate and tape that in opposite ends by placing the comb
6. Pour the gel in gel plate evenly without getting air bubbles and make it solidify
7. Remove comb from the gel
8. Take parafilm and add 3ul of loading dye and 1ul of DNA sample on dye
9. Load them in the Wells
10. Always add a ladder for our knowledge so that we can easily find out the size.

## SEQUENCING:

DNA sequencing is the process of determining the nucleic acid sequence – the order of nucleotides in DNA. It includes any method or technology that is used to determine the order of the four bases: adenine, guanine, cytosine, and thymine. The advent of rapid DNA sequencing methods has greatly accelerated biological and medical research and discovery

Types of sequencing

1. Sanger sequencing
2. Next generation sequencing

**Fragment analysis** is a process were we can read size and length of the fragment

**GENETIC ANALYZER :**

Genetic analyzers are automated systems capable of sequencing DNA or analyzing fragments for a variety of applications. In capillary electrophoresis-based systems, DNA fragments bound to probes migrate through a polymer and the fluorescence emissions are measured.

DNA sequencing has 3 steps

1. clean up

- Gel purification
- Column purification
- EXOSAP
- Direct sequencing

2.Cycle sequencing PCR

3.Post PCR clean up

**EXOSAP CLEAN UP:**

Among all other cleanup reagents, ExoSAP-IT single-step PCR is the best reagent which uses two hydrolytic enzymes. And the two hydrolytic enzymes, Shrimp Alkaline Phosphatase which are added together in certain ratio in order to form a buffer solution which is used to eliminate undesired or not required dNTP’s and

primers from PCR products. Exonuclease I eliminates single-stranded primers and any other unrelated single-stranded DNA developed in the PCR and leaves no residuals. Where as SAP eliminates the rest of dNTPs in the PCR batter or PCR products.

ExoSAP-IT reagent can delivers its maximum potential in treating PCR products ranging their sizes from 100bp to 20kb and eliminates the not required or irrelevant primers and nucleotides. To speed up the process of getting results from PCR buffers we add ExoSAP-IT reagent straight to the PCR products. No buffer exchanges are needed to activate the function or the cleaning process of PCR products by using ExoSAP-IT reagent. To inactivate the effect of ExoSAP-IT the PCR buffer is heated to 80°C continuously for 7 minutes straight. This complete process id used to eliminate the unwanted primers and nucleotides from DNA which is used for further analysis.

**CYCLE SEQUENCING PCR :**

Cycle sequencing - It is a technique to improve the sensitivity of DNA sequencing process by allowing to use tiny amounts of DNA starting material and this technique is best known for its robust and easy performance. And the whole process is achieved by applying temperature cycling process which is completely identical to polymerase chain reaction.

| Sno | Reagents |
|---|---|
| 1 | Exosap purified PCR product |
| 2 | Big dye |
| 3 | Sequencing buffer |
| 4 | H2O |

| | |
|---|---|
| 5 | Forward primer |

Sequencing PCR conditions:

1. 96°C for 30secs
2. 55°C for 7secs
3. 58°C for 4min
4. 12°C Hold

## POST PCR CLEANUP:

1. Remove ExoSAP-IT reagent from -20°C freezer and keep on ice throughout this procedure.

2. Mix 2µl of a post-PCR reaction product with 0.8µl of 70%ExoSAP-IT reagent for a combined 3µl reaction volume and spin for 30 sec.

3. In a PCR machine, incubate at 37°C denaturation to degrade remaining primers and nucleotides, followed by 80°C annealing to inactivate ExoSAP-IT reagent.

4. Add 10µl water and 2µl 125mm EDTA .2 µl of 3M sodium acetate +50µl ethanol

5. Incubate for 15min
6. Centrifuge at 12000g for 20 min.
7. Discard the supernatant, add 250 µl 70%ethanol.
8. Centrifuge at 12000g for 10min.
9. Discard the supernatant.
10. Air-dry for 20min

## SAMPLE LOADING

1. Add10 µl of HIDI formamide to the sample containing tube.
2. Mix them
3. Spin down using centrifuge for 2 min
4. Keep the plate for denaturation
5. Snap chill for 30 sec to 1 min
6. Spin down for 1 min
7. Load the plate on to the sequencer

## DATA COLLECTION SOFTWARE

1. Instrument and the system should be switched on simultaneously
2. Open the software in the system
3. Open dash board and check the consumables which mean anode buffer, cathode buffer and polymer.
4. We should open the instrument and set the instrument with sample plate
5. In the system we should create the plate
6. Assign all the plate contents
7. Link the plate
8. Create injection list, review and modify
9. We should start the run
10. We can see different blocks in the system like

- Eletropherograms
- Annotation
- Sequence
- Raw data

- EPT

11. In electropherogram we can see the sequence
12. In annotation we can see the average signal intensity more than 250 is good
13. Noise is even can be seen below 15 is good
14. Length of the detector can be seen
15. In the sequence we can see the quality value it should be more than 20.
16. Raw data will say you the read length
17. EPT electrophoresis process temperature uses for the trouble shoots no abnormal line should be seen once after the stability
18. Where we can see the colors beside our samples in a block green color indicates good results and blue even is acceptable and rest all other colors are with warning.
19. These colors shows the quality of the data

**CODON CODE ALIGNER:**

Codon code aligner is a software tool used to assemble the sequence, aligning the sequence and contig editing, and mutation detection, available for windows and Mac OS X.

Aligner is compatible with Phred-Phrap and fully supports sequence quality scores, while offering a familiar, easy to learn user interface as shown in the fig below

In codon code aligner

Import sequence from files

Import referral sequence

Assemble the sequence

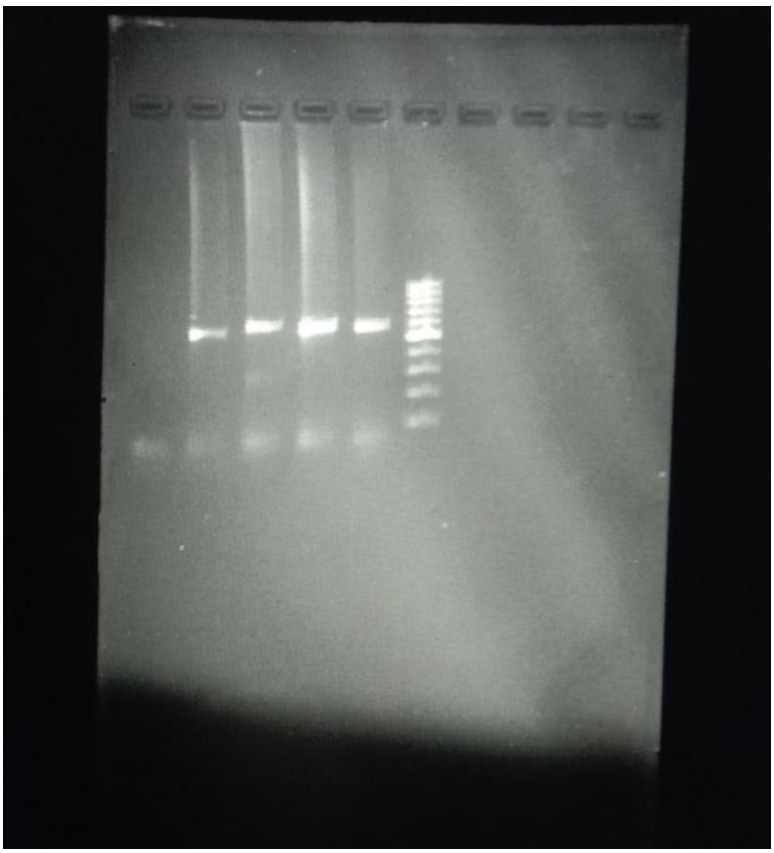
Contig the sequence and trace the sequence

Find heterozygous mutations (insertions or deletions)

Analyse your results.

**RESULT**

**Gel electrophoresis:**



DNA bands of Pcr products on gel electrophoresis

- Lane 1 to 5 are DNA bands which are 500 base pairs and lane 6 is ladder which is 1000 base pairs

Total number of samples are 10

From 10 samples we found 3 different variants

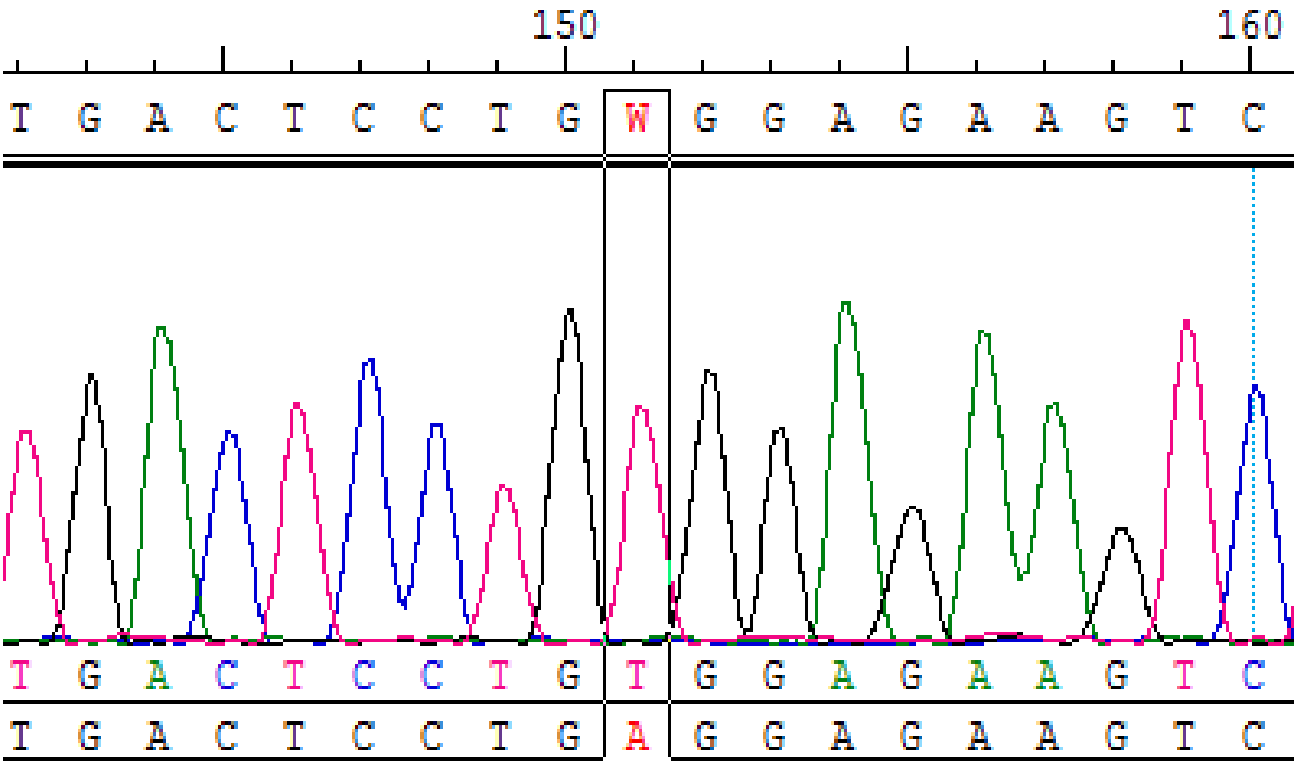
| Sample no | Variant | Type of variant | Type of mutation | Position | Clinical Suspicion | Mutational Diagnosis |
|---|---|---|---|---|---|---|
| 1 | c.20A>T | Pathogenic | Homozygous | Exon 1 | Sickle cell | Sickle cell |
| 2 | c. 92+5G>C | Pathogenic | Homozygous | Intron 1 | Sickle cell and thalassemia | Thalassemia |
| 3 | c.20A>T | Pathogenic | Heterozygous | Exon 1 | Sickle cell | Sickle cell |
| 4 | c.20A>T | Pathogenic | Homozygous | Exon 1 | Sickle cell | Sickle cell |
| 5 | c.20A>T c.47G>A | Pathogenic | Compound Heterozygous | Exon 1 | Thalassemia | Sickle thalassemia |
| 6 | c.20A>T | Pathogenic | Homozygous | Exon 1 | Sickle cell | Sickle cell |
| 7 | c.20A>T | Pathogenic | Homozygous | Exon 1 | Sickle cell | Sickle cell |
| 8 | c.20A>T | Pathogenic | Homozygous | Exon 1 | Sickle cell | Sickle cell |
| 9 | c.20A>T | Pathogenic | Homozygous | Exon 1 | Sickle cell | Sickle cell |
| 10 | c.20A>T | Pathogenic | Homozygous | Exon 1 | Sickle cell | Sickle cell |

**Sample 1**

Variant : c20A>T

Type of mutation : **Homozygous**

Position: Exon 1



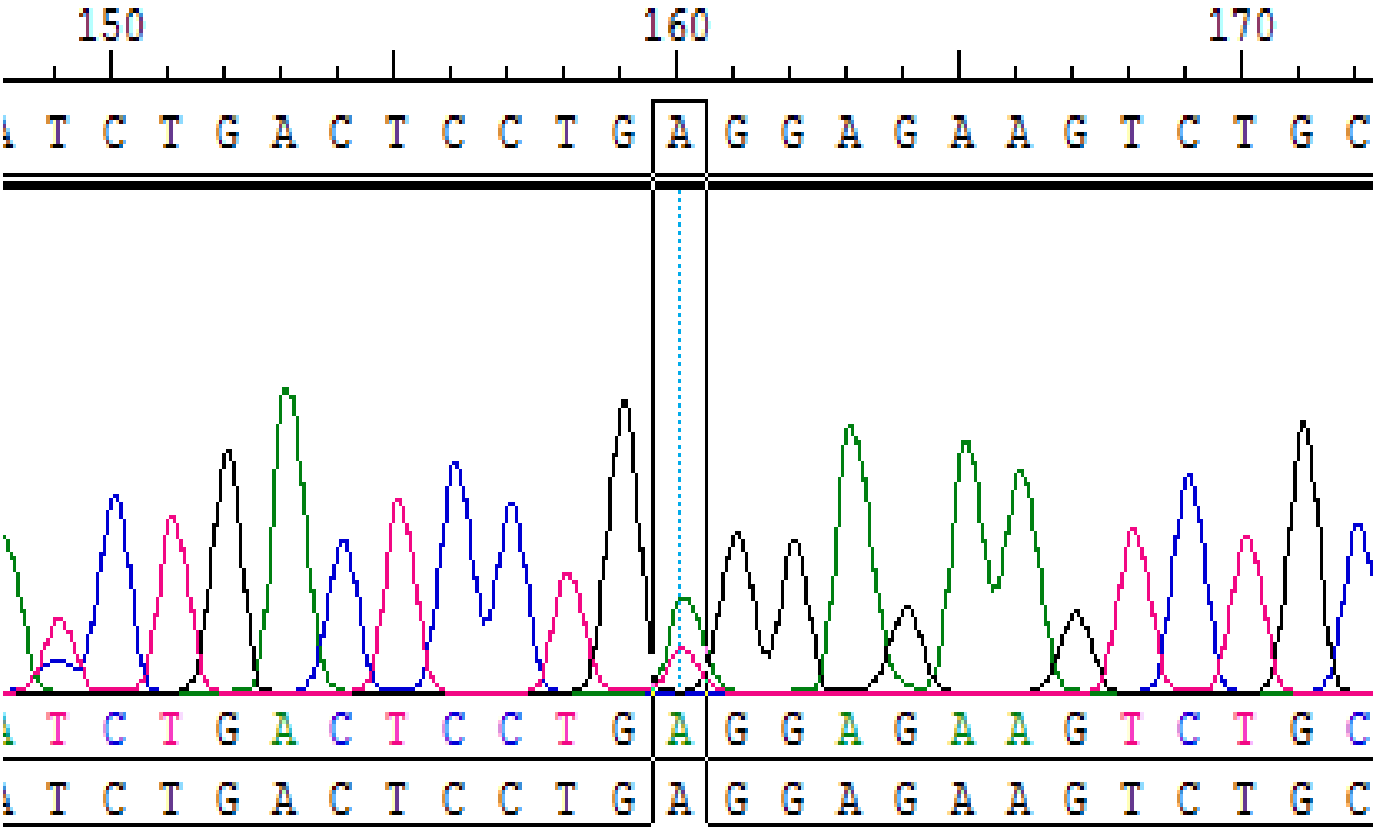

**Mutational diagnosis : sickle cell**

**Sample 3**

Variant : c20A>T

Type of mutation : **Heterozygous**

Position: Exon 1



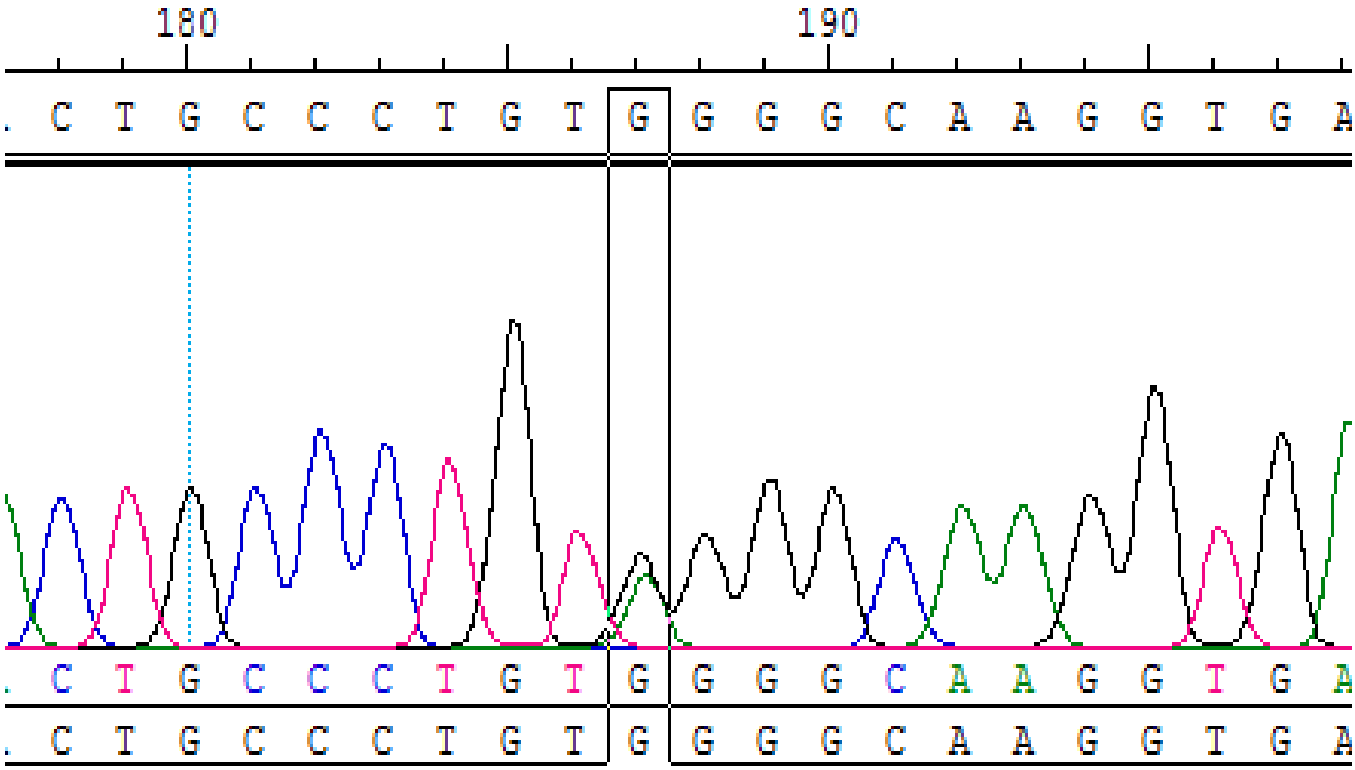

**Mutational diagnosis : sickle cell**

**Sample 5**

Variant : c20A>T

Type of mutation : **Compound Heterozygous**

Position: Exon 1




**Mutational diagnosis : sickle thalassemia**

Genotypic frequency and allelic frequency of variants

| | | Frequency |
|---|---|---|
| Genotypic | AA<br>AT<br>TT<br>GG<br>CC<br>GC<br>GG<br>TT<br>GT | |
| Allelic | A<br>T<br>G<br>C | |

# DISCUSSION:

Sickle cell disease is a hereditary hemoglobinopathy resulting from Inheritance of a mutant version of the β-globin gene (βA) on Chromosome 11. This condition is common in tribal population of India. Process of Mutational diagnosis of sickle cell anaemia includes isolation of DNA in which DNA is isolated and quantification of DNA is done by quit flourometer, which is followed by Polymerase chain reaction (PCR) were we include primers, dntps. After PCR gel electrophoresis conducted which shows base pairs through bands, there are 500 base pairs found in these samples.

Sequencing of the samples shows 3 different variants they are homozygous, heterozygous, compound heterozygous. By the analysis of variant, mutation and position mutational diagnosis is found.

# CONCLUSION:

Sickle cell disease is a chronic, debilitating disorder with a myriad of symptoms that make disease treatment challenging. While there is a need for new treatment for sickle cell disease, especially for disease modifying agents, there is also a need to explore new approaches for improving treatment with existing modalities. Preventive measures particularly in disease endemic area must be taken such as premarital genetic counseling and screening. Future research must be focused on decreasing the number of crises and blood transfusion through new remedies having easy availability, less cost and minimum side effects. The tribal people of Central and Southern India had a geographical unicentric origin and had unicentric origin of the mutated gene when these tribal populations were in direct contact and underwent panmixia or gene flow. But now they are dispersed and live distantly isolating themselves and maintain strict endogamy which has lead to high frequency of HbS gene.

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