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Sickle cell disease: An introductory supplement

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HISTORY OF SICKLE CELL DISEASE

Sickle cell first reported by James B Herrick[1] (United States,) in red blood cells, year 1910. The disease was named sickle-cell anaemia by Verne Mason in 1922. Pauling[2] described sickle cell anaemia as a molecular disease and showed that it had different electrophoretic properties from normal human haemoglobin. This was the first time a genetic disease was linked to a mutation of a specific protein, a milestone in the history of molecular biology, and it was published in their paper "Sickle Cell Anemia, a Molecular Disease". Subsequently, this change in electrophoretic mobility was found to be due to the substitution of the glutamic acid residue at position 6 by valine in the beta chain of the haemoglobin molecule (β6 Glu→Val) [3]. The first cases of sickle cell anaemia in an Indian of 22 years age, born of Indian parents in Durban was reported by Berk and Bull[4] from Cape Town. Sickle cell tarit in South India was first described by Lehmann and Cutbush[5] among the original tribes of Nilgiri Hills. In the same year Dunlop and Mazumder[6] reported five cases of sickle cell trait and three presumptive cases of sickle cell anaemia among the tea garden labourers of Upper Assam, originating from the tribal population of Orissa and Bihar. HbS gene is widespread among many tribal and non tribal populations of India.

PATHOPHYSIOLOGY

Sickle-cell disease (SCD) is an autosomal recessive genetic blood disorder with incomplete dominance, characterized by red blood cells that assume an abnormal, rigid, sickle shape. Sickling decreases the cells' flexibility and results in a risk of various complications. The sickling occurs because of a mutation in the hemoglobin gene. Life expectancy is shortened, with studies reporting an average life expectancy of 42 in males and 48 in females[7]. Sickle-cell anaemia is caused by a point mutation in the β-globin chain of haemoglobin, causing the hydrophilic amino acid glutamic acid to be replaced with the hydrophobic amino acid valine at the sixth position. The β-globin gene is found on the short arm of chromosome 11. The association of two wild-type α-globin subunits with two mutant β-globin subunits forms haemoglobin S (HbS). Under low oxygen conditions the absence of a polar amino acid at position six of the β-globin chain promotes the non-covalent polymerisation of haemoglobin, which distorts red blood cells into a sickle shape and decreases their elasticity.

SICKLE AND MALARIA

Sickle cell disease, usually presenting in childhood, occurs more commonly in people from

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parts of tropical and sub-tropical regions where malaria is or was common. One-third of all indigenous inhabitants of Sub-Saharan Africa carry the gene, because in areas where malaria is common, there is a fitness benefit in carrying only a single sickle-cell gene (sickle cell trait). Those with only one of the two alleles of the sickle-cell disease, while not totally resistant, are more tolerant to the infection and thus show less severe symptoms when infected[8].

NOMENCLATURE AND SUB TYPES

Sickle-cell anemia is the name of a specific form of sickle-cell disease in which there is homozygosity for the mutation that causes HbS. Sickle-cell anaemia is also referred to as HbSS, SS disease, or haemoglobin S. In heterozygous people, who have only one sickle gene and one normal adult haemoglobin gene, it is referred to as HbAS or sickle cell trait. Other, rarer forms of sickle-cell disease include sickle-haemoglobin C disease (HbSC), sickle hemoglobin D disease (HbSD), sickle beta-plus-thalassaemia (HbS/β^+), Sickle hemoglobin E disease (HbSE) and sickle beta-zero-thalassaemia (HbS/β^0). These other forms of sickle-cell disease are compound heterozygous states in which the person has only one copy of the mutation that causes HbS and one copy of another abnormal haemoglobin allele. The term disease is applied, because the inherited abnormality causes a pathological condition that can lead to death and severe complications. Not all inherited variants of haemoglobin are detrimental, a concept known as genetic polymorphism.

GENETICS AND MOLECULAR ORGANIZATION OF SICKLE CELL

Sickle cell results from a specific point mutation (A > T) causing the substitution of glutamic acid by valine amino acid at six position in the beta-globin chain in chromosome no. 11.

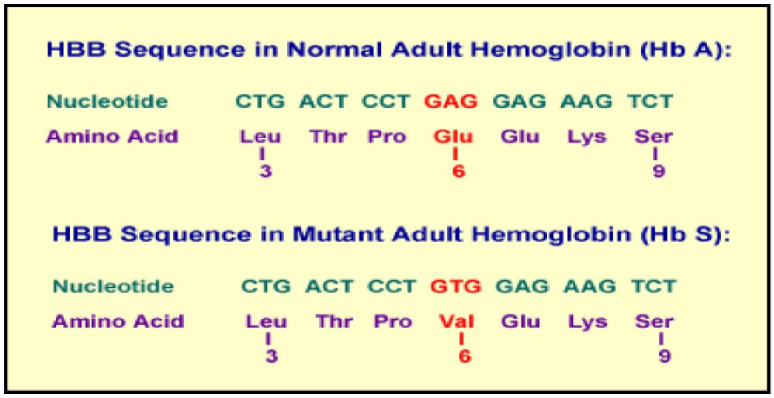

Figure 1 : Changing of amino acid in polypeptide chain

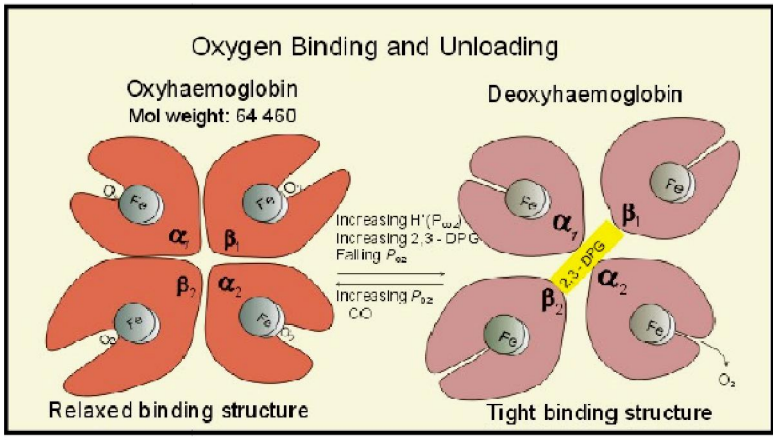

Figure 2 : Oxygenated and deoxygenated state of haemoglobin

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Human Hemoglobins		
Embryonic hemoglobins	**Fetal hemoglobin**	**Adult hemoglobins**
Gower 1- zeta(2), epsilon(2) Gower 2-alpha(2), epsilon (2) Portland- zeta(2), gamma (2)	hemoglobin F-alpha(2), gamma(2)	hemoglobin A- alpha(2), beta(2) hemoglobin A2- alpha(2), delta(2)

Figure 3 : Variant of hemoglobin

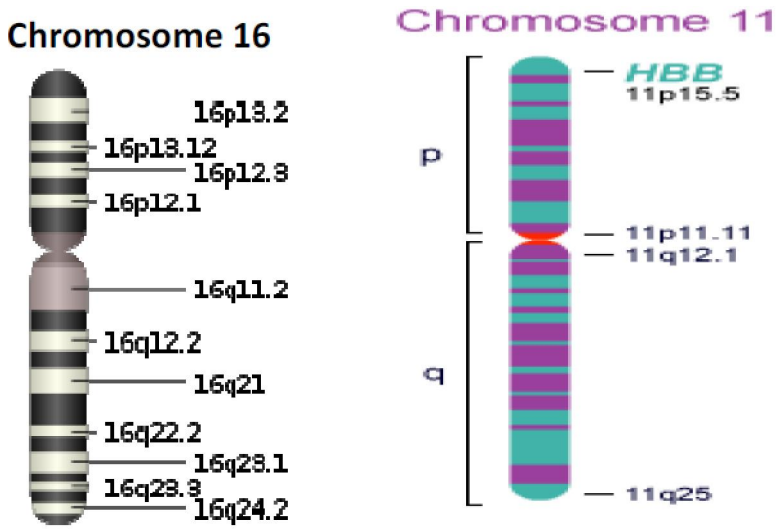

Figure 4 : Chromosomes involve in Hemoglobin formation

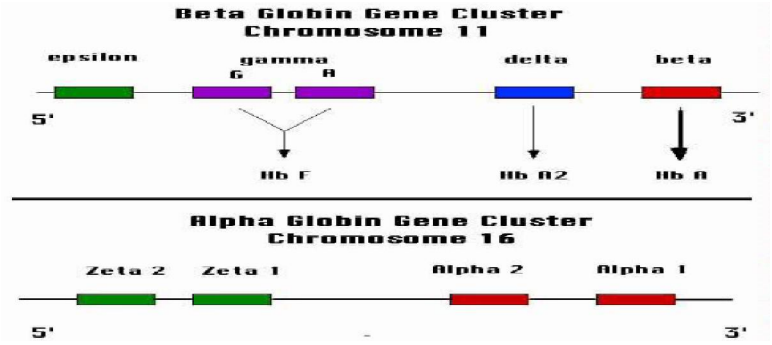

Figure 5 : Diagrammatic Representation of beta globin and alpha gene cluster in chromosome 11 and 16 respectively

Hemoglobin-Hemoglobin (Hb) is responsible for transport of oxygen from lungs to the tissue and of CO_2 from tissue to the lungs. Hb (MW 64,500 dalton) is composed of heam (consisting of iron and porotoporphyrin) and globin. The globin portion of the molecule consists of four (or two pair of) polypeptide chains. One heam group is bound to each polypeptide chain.

The relative proportions of different Hemoglobin:

Adult HbA-97%, HbA2-2.5%, And HbF 0.5%

Newborns HbF-80% And Hba- 20%

Beta globin gene found in chromosome No.11 while alpha globin gene found in chromosome 16, assembling of both cluster make haemoglobin

SIGN AND SYMPTOMS

Sickle cell crisis- Most episodes of sickle cell crises last between five and seven days included

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C. Order of genes in α-globin gene cluster/

5 -ζ-ψ ζ - ψ α2 – ψ α1- α2- α1 -3' /

D. Alpha genotype

Normal αα/αα
Silent carrier - α/αα
Minor -α/-α --/αα
Hb H disease --/-α
Barts hydrops fetalis --/--

Order of genes in β-globin gene cluster

5'-ε-Gγ-Aγ- ψ β-δ-β-3'

Beta genotype

Normal β/β
Minor β/β^{+} β/β^{0}
Intermedia β^{0}/β^{+} β^{+}/β^{+}
Major β^{0}/β^{0}

E. Beta genotype with Sickle gene

Genotype	Full Name	Abbreviation
β^{s}/β^{s}	Sickle cell anemia	HbSS
β^{s}/β^{c}	Sickle cell disease-C	HbSC
β^{s}/β^{o}	Sickle cell β^{0} thalassemia	HbS β^{o} thal
β^{s}/β^{+}	Sickle cell β^{+} thalassemia	HbS β^{+} thal
β^{s}/β^{D}	Sickle cell D disease	HbSD
β^{s}/β^{A}	Sickle trait	HbSA

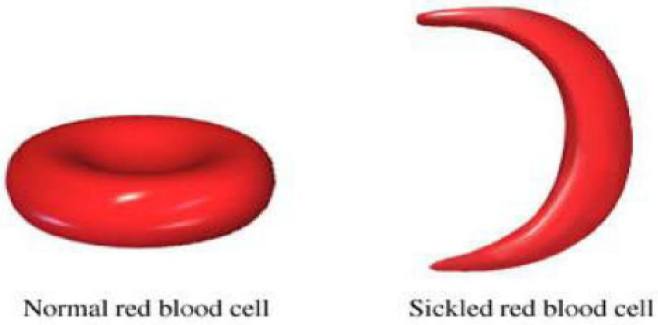

Figure 6 : Physical structure of Normal and sickle shaped Red blood cell

vaso-occlusive crisis, aplastic crisis, sequestration crisis, haemolytic crisis and others

Vaso-occlusive crisis

The vaso-occlusive crisis is caused by sickle-shaped red blood cells that obstruct capillaries and restrict blood flow to an organ, resulting in ischaemia, pain, necrosis and often organ damage. The frequency, severity, and duration of these crises vary considerably.

Splenic sequestration crisis

Because of its narrow vessels and function in clearing defective red blood cells, the spleen is frequently affected. It is usually infracted before the end of childhood in individuals suffering from sickle cell anaemia. This autosplenectomy increases the risk of infection from encapsulated organisms[9, 10]. Splenic sequestration crises are acute, painful enlargements of the spleen. The sinusoids and gates would open at the same time resulting in sudden pooling of the blood into the spleen and circulatory defect leading to sudden hypovolaemia. The abdomen becomes bloated and very hard. Splenic sequestration crises are considered an emergency. If not treated, patients may die within 1–2 hours due to circulatory failure. Management is supportive, sometimes with blood transfusion. These crises are transient; they continue for 3–4 hours and may last for one day.

Aplastic crisis

Aplastic crises are acute worsening of the patient's baseline anaemia, producing pallor, tachycardia, and fatigue. This crisis is triggered by parvovirus B19, which directly affects erythropoiesis by invading the red cell precursors and multiplying in them and destroying them. Parvovirus infection nearly completely prevents red blood cell production for two to three days. This crisis takes 4 days to one week to disappear. Most patients can be managed supportively; some need blood transfusion.

Haemolytic crisis

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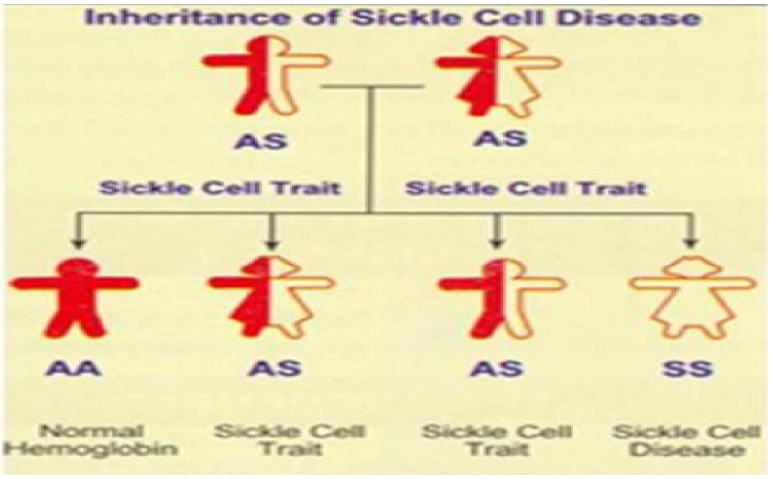

Figure 7 : Diagrammatic representation of inheritance of sickle gene

TABLE 1 : Prevalence of sickle cell in some states of India[32].

State	Prevalence of sickling (%)
Andhra Pradesh	0-34.6
Bihar	0-0.6
Gujarat	0-30
Karnataka	0-25
Kerala	0-29.7
Maharashtra	0-45.4
Madhya Pradesh	0-48.5
Orissa	0-12.4
Tamil Nadu	0-35.3
Utter Pradesh	0-32.6
West Bengal	0-1.1

TABLE 2 : State wise distribution of sickle sub types in India[33]

	Sickle sub- types		
States	**HbSS N=60**	**HbSβ-thal. N=75**	**HbSD N=15**
Orissa	13(21.66%)	14(18.66 %)	2(13.33 %)
Bihar	11(18.33%)	10(13.33 %)	1(6.66 %)
Jharkhand	8(13.33%)	6(8%)	2(6.66 %)
W. Bengal	7(11.66%)	7(9.33 %)	4(26.66 %)
Madhya Pradesh	5(8.33%)	6(8%)	-
Chhattisgarah	7(11.66%)	8(10.66 %)	-
Karnataka	6(10%)	5(6.66%)	-
Delhi	3 (5%)	1(1.33 %)	-
Rajasthan	-	7(9.33 %)	2(6.66 %)
Uttar Pradesh	-	4(5.33%)	-
Hariyana	-	3(4 %)	-
Punjab	-	4(5.33%)	4(26.66%)

Haemolytic crises are acute accelerated drops in haemoglobin level. The red blood cells break down at a faster rate. This is particularly common in patients with co-existent G6PD deficiency. Management is supportive, sometimes with blood transfusions.

COMPLICATIONS

- Overwhelming post splenectomy infection, which is due to functional asplenia, caused by encapsulated organisms such as *Streptococcus*

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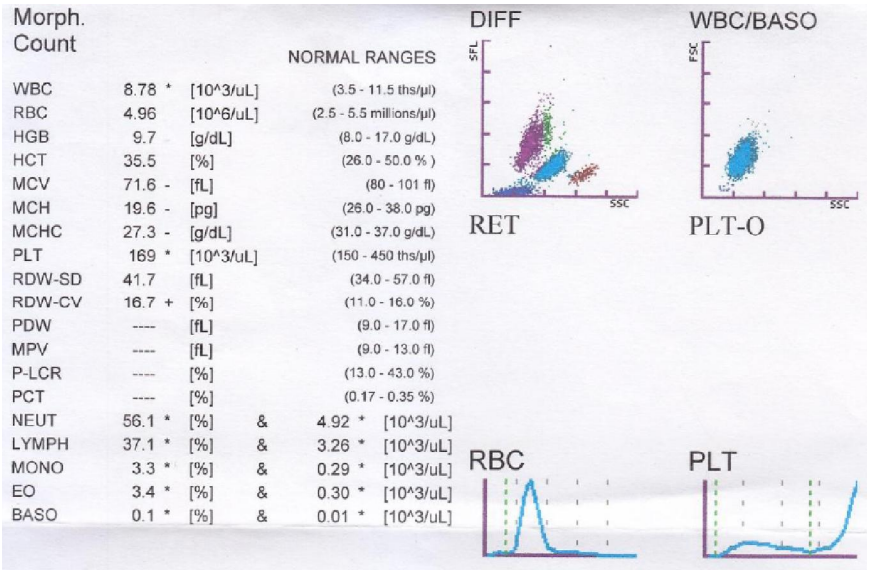

Figure 8 : Complete blood count of sickle cell patients

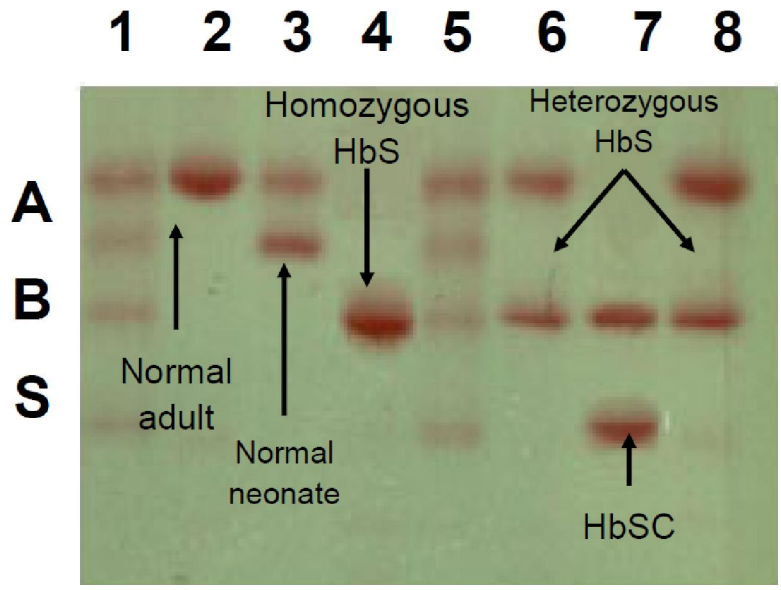

Figure 9 : Hemoglobin electrophoresis

pneumoniae and *Haemophilus influenzae*.

- Stroke, which can result from a progressive narrowing of blood vessels, preventing oxygen from reaching the brain. Cerebral infarction occurs in children and cerebral haemorrhage in adults.
- Cholelithiasis and cholecystitis, which may result from excessive bilirubinproduction and precipitation due to prolongedhaemolysis.
- Avascular necrosis of the hip and other major joints, which may occur as a result of ischaemia[11].
- Decreased immune reactions due tohyposplenism[12].
- Priapism and infarction of the penis[13].
- Acute papillary necrosis in the kidneys.
- Leg ulcers[14].
- Chronic renal failure due to sickle cell nephropathy manifests itself with hypertension , proteinuria, haematuria and worsened anaemia. If it progresses to end-stage renal failure, it carries a poor prognosis.
- Osteomyelitis (bacterial bone infection); the most common cause of osteomyelitis in sickle cell disease is *Salmonella* followed by *Staphylococcus aureus* and Gram-negative enteric bacilli perhaps because intravascular sickling of the

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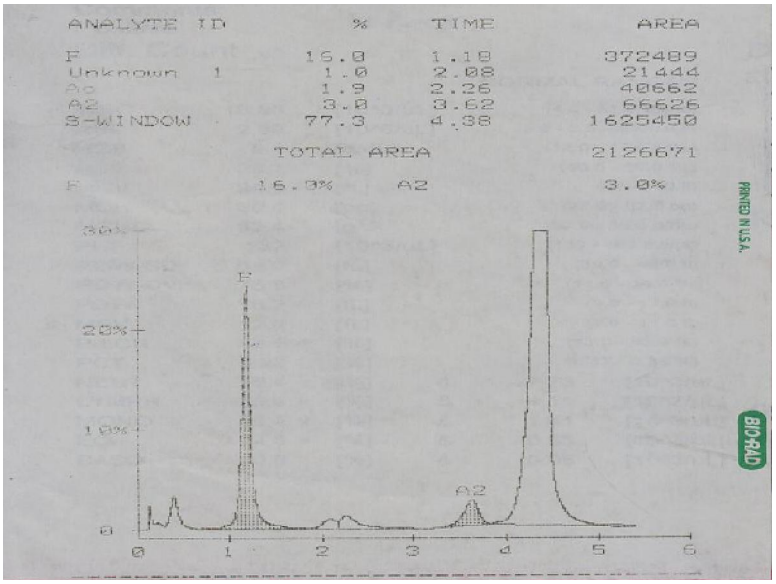

Figure 10 : Chromatogram of sickle cell patients

bowel leads to patchy ischaemic infarction[15].

INHERITANCE

Sickle-cell conditions are inherited from parents in much the same way as blood type, hair colour and texture, eye colour, and other physical traits. The types of haemoglobin a person makes in the red blood cells depend on what haemoglobin genes are inherited from his parents. If one parent has sickle-cell anaemia (SS) and the other has sickle-cell trait (AS), there is a 50% chance of a child's having sickle cell disease (SS) and a 50% chance of a child's having sickle-cell trait (AS). When both parents have sickle-cell trait (AS), a child has a 25% chance (1 of 4) of sickle-cell disease (SS), as shown in the diagram.

EPIDEMIOLOGY

The highest gene frequencies are found in equatorial Africa where it exceeds 20% in the Cameroon, Guinea, Zaire, Uganda and Kenya. In Quatif Ouses of Saudi Arabia and parts of India, HbS is known to occur at frequencies of upto 20%. Modiano[16] reported HbS in Nepal with a gene frequency of 5%. Around the Mediterranean (the coast of North Africa, Turkey, Lebanon, Syria, Greece and Portugal), in the Middle East and in Iran it occurs generally at frequencies of less than 5%. Frequencies of HbS vary substantially from one group to another in these regions (14% in Eti-Turks of Turkey, 25% in Khazramahs of Syria).

United states

The prevalence of the disease in the United States is approximately 1 in 5,000, mostly affecting Americans of Sub-Saharan African descent, according to the National Institutes of Health. In the United States, about 1 out of 500 African-American children born will have sickle cell anaemia.

Africa

Three quarters of sickle-cell cases occur in Africa. A recent WHO report estimated that around 2% of newborns in Nigeria were affected by sickle cell anaemia, giving a total of 150,000 affected children born every year in Nigeria alone. The carrier frequency ranges between 10% and 40% across equatorial Africa, decreasing to 1–2% on the North African coast and <1% in South Africa[17].

France

In Europe, the highest prevalence of the disease has been observed in France. As a result of population growth in African-Caribbean regions of overseas France, and now immigration essentially from North and sub-Saharan Africa to mainland France, sickle cell disease has become a major health problem in France.[18] SCD has become the most com-

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mon genetic disease in this country, with an overall birth prevalence of 1/2,415 in mainland France[19].

United kingdom

In United Kingdom, more than 200 babies are born annually with SCD.

Middle east

About 6,000 children are born annually with SCD, at least 50% of these in Saudi Arabia, especially in Qatif City.

India

Sickle cell disease is prevalent in many parts of India, where the prevalence has ranged from 9.4 to 22.2% in endemic areas[20]. India caters to nearly 20 million people with sickle cell disease[21]. The sickle cell gene in India was first described among tribal groups in South India[5], which spread the wrong message that the disease is confined to tribal people. But the recent data unfolds that the disease is not restricted only to tribal belt but is widely prevalent and has penetrated different castes and communities in country[22]. It is widely recognized especially in the central parts of India[23]. The highest frequency of sickle cell gene in India is reported in Orissa followed by Assam, MP, UP, Tamilnadu and Gujarat[24]. The average frequency of sickle cell disease in India is 4.3% and that of Orissa is as high as 9.1%[25]. Children comprised 52% of sickle cell patients and the three predominant forms of the disease (SS, S and SD) are clinically and haematologically indistinguishable.[26] Studies from Orissa report the incidence of SCD in hospitalized pediatric patients to be 6.42% and 11.1%[27,28]. Central Maharashtra is reported to be in the sickle cell belt[29]. In Central India, prevalence of sickle cell trait (SCT) has been reported to be 11.1%[30]. A community survey of the rural population in this area, based on sickling test, showed a prevalence of 5.5%, of which on electrophoresis, 5.86% had HbSS and 94.1% had HbAS[31]. The prevalence of sickling in some states of India is depicted in Table Below.

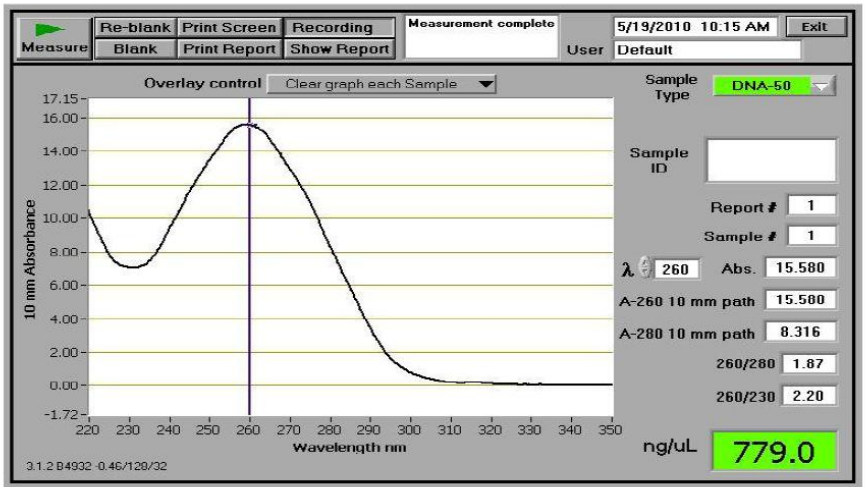

Figure 11 : DNA concentration by nano drop method

TABLE 3 : Reaction mixture

S.No.	Chemical	Amount(28µl)
1.	1x Frankfurt buffer	20µl
2.	Fw primer	0.5µl
3.	Rw primer	0.5µl
4.	$MgCl_2$	1 µl
5.	DMSO	3 µl
6.	Taq polymerase	0.5 µl
7.	DNA Sample	2.5 µl

DIAGNOSIS

In HbSS, the full blood count reveals haemoglobin levels in the range of 6–8 g/dL with a high reticulocyte count (as the bone marrow compensates for the destruction of sickle cells by producing more red blood cells). In other forms of sickle-cell disease, Hb levels tend to be higher. A blood film may show features of hyposplenism (target cells and Howell-Jolly bodies).

A. Complete blood counts (CBC)

A complete blood count (CBC) is a common blood test used to evaluate overall health and detect a wide range of disorders, including sickle disorder. A complete blood count (CBC) gives important information about the kinds and numbers of cells in the blood, especially red blood cells, white blood cells, and platelets. Complete blood count and red cell indices can be measured by automated cell analyzer. Giemsa-stained peripheral blood smears can be examined for red cell morphology.

Interpretation- HGB & red cell indices low in sickle patients

Normal cut-off value is following

WBC: 3.5-11.5 ths/µl. RBC:2.5-5.5 Million/µl. HGB:8.0-17.0 g/dL. HCT:26.0-50.0 %.

MCV:80-101 fl. MCH :26.0-38.0 pg. MCHC:31.0-37.0 g/dL. PLT:150-450 ths/µl.

B. Sickling & solubility test

Sickling of the red blood cells, on a blood film, can be induced by the addition of sodium metabisulfite. The presence of sickle haemoglobin can also be demonstrated with the sickle solubility test. A mixture of haemoglobin S (Hb S) in a reducing solution (such as sodium dithionite) gives a turbid appearance, whereas normal Hb gives a clear solution.

Hemoglobin electrophoresis

Abnormal hemoglobin forms can be detected on hemoglobin electrophoresis, a form of gel electrophoresis on which the various types of hemoglobin move at varying speeds. Sickle cell hemoglobin (HbS) and hemoglobin C with sickling (HbSC);the two most common forms can be identified from there.

D. Screening of sickle cell patients by HPLC

A cation- exchange high performance liquid chromatography (HPLC) can be used for quantification of HbA2, HbF, HbA0, HbD and HbS for detection of HbS sub-type.

Interpretation

SA -When HbS % < 50

Sβ -When HbS % > 50 & HbF high and HbA2 raised

SS- When HbS % >50 and HbF raised

E. Molecular analysis of disease

Isolation of DNA- DNA can be isolated from leukocyte collected from Venus blood in anticoagulant coated vials. Phenol chloroform or kit method can be used to isolate DNA according to protocol.

Quantification of DNA

The quantity of DNA can be estimated by spectrophotometer by nano drop method. OD at 260/280 ratio 1.7-1.9 suggest that DNA is pure. According to the concentration the amount of DNA can be decided which were added in the PCR reaction mixture.

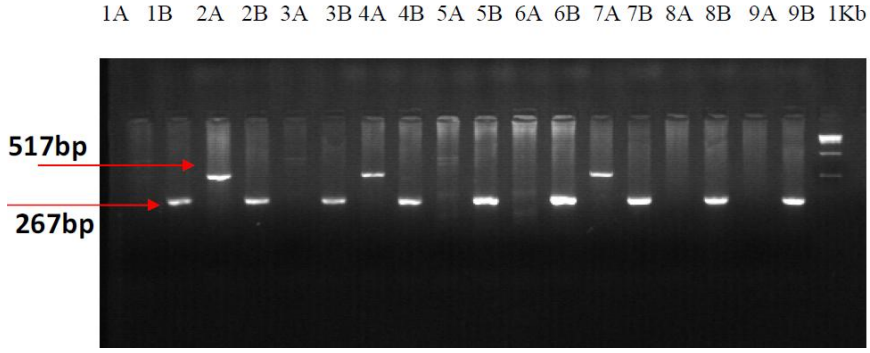

Figure 12 : Gel picture of sickle cell gene (Lane 1,3,5,6,8,9 homozygous and 2,4,7 heterozygous)

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Genotypic detection of sickle cell by allele specific PCR

Primer sequence[34]

WT-AS (52 -ATG GTG CAC CTG ACT CCT GA-32) WT- Fw control

CP517 (52 -CCC CTT CCT ATG ACA TGA ACT-32) Rw control

MUT-AS (52 -CAG TAA CGG CAG ACT TCT CCA-32) Fw mutant

MUT-CP267 (52 -GGG TTT GAA GTC CAA CTC CTA-32) Rw mutant

PCR program for amplification

- Incubate at 95^0C for 00 :02:00
- Incubate at 95^0C for 00 :00:30
- Incubate at 65^0C for 00 :00:30
- Incubate at 72^0C for 00 :00:30
- Cycle to step 2 for 30 more times.
- Incubate at 72^0C for 00 :05:00
- Incubate at 10^0C for 00 :15:00

Confirmation of amplification

Amplified products were run on 2% agarose gel

Gel picture illustrated the homozygous and heterozygous condition below.

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