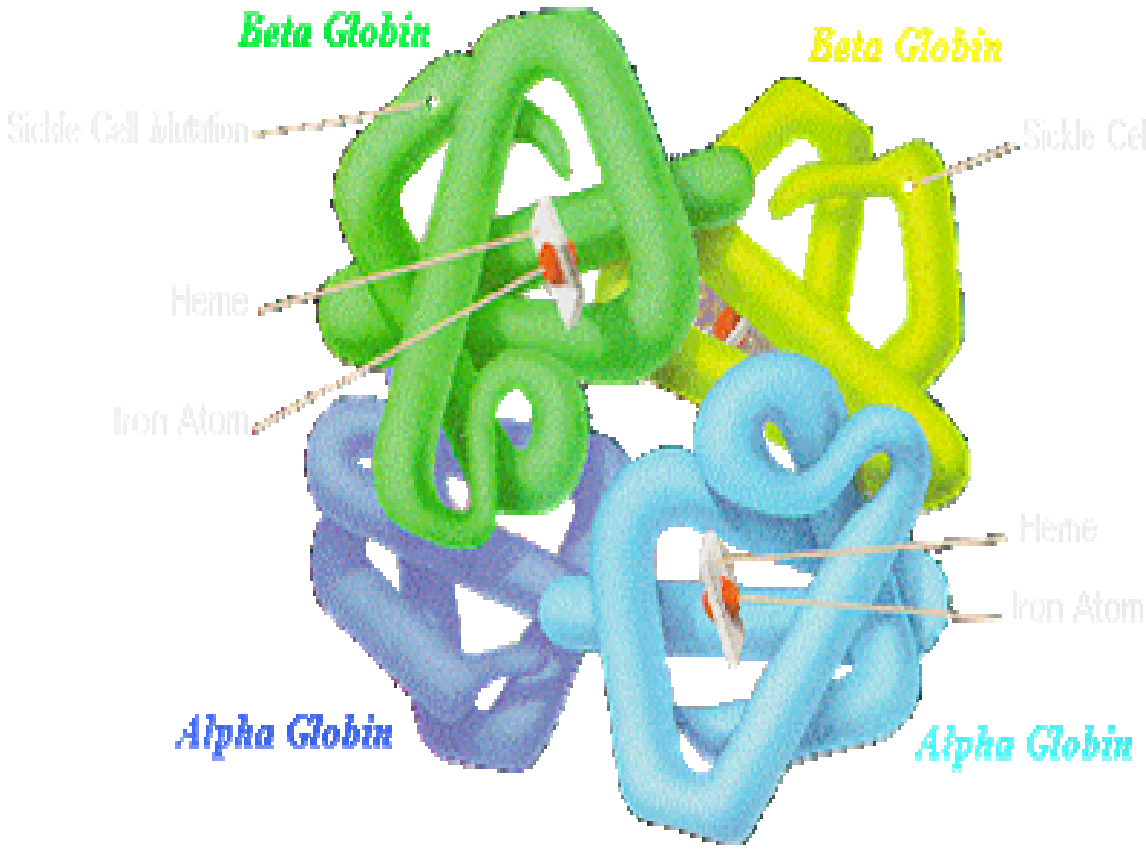
# Hemoglobin Profile of Anemic Ethiopian Patients Attending Tikur Anbessa Specialized Hospital

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# Addis Ababa University
# School of Graduate Studies

## Hemoglobin profile of Anemic Ethiopian Patients attending Tikur Anbessa Specialized Hospital

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***A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirement of the Degree of Master of Science in Biochemistry***

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## Abbreviations

| | |
|---|---|
| AAU | Addis Ababa University |
| 2,3-BPG | 2,3-Bisphosphoglycerate |
| CBC | Complete blood count |
| dl | deciliter |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylene diamine tetra acetic acid |
| EPO | Erythropoietin |
| fl | Femtoliter |
| G6PD | Glucose- 6 -phosphate dehydrogenase |
| Hb | Hemoglobin |
| HbA | Adult Hemoglobin |
| HbF | Fetal Hemoglobin |
| HbS | Sickle cell hemoglobin |
| HCT | Hematocrit |
| HPFH | Hereditary persistence of fetal hemoglobin |

| | |
|---|---|
| HPLC | High pressure liquid chromatography |
| IEF | Isoelectric focusing |
| MBA | Megaloblastic anemia |
| MCV | Mean cell volume |
| MCH | Mean cell hemoglobin |
| MCHC | Mean cell hemoglobin concentration |
| RBC | Red Blood Cell |
| RPM | Revolution per minutes |
| RDW | Red cell volume Distribution Width |
| PAGE | Polyacrylamide gel electrophoresis |
| Pg | Picogram |
| TASH | Tikur Anbessa Specialized Hospital |
| TEMED | N,N,N',N', Tetramethylenediamine |
| WBC | White blood cells |

# 1. Abstract

Hemoglobinopathies are inherited abnormalities of hemoglobin molecule that affect the production, structure and function of Hb. Hb is the protein of the red blood cells that binds oxygen reversibly and transport it from the lungs to the tissue and return carbon dioxide from the tissue back to the lungs. Hemoglobin genes are subject to mutations that alter the synthesis and structure of the protein. These diseases are common lethal genetic defects in some part of the world. This study was undertaken to find out the hemoglobin profile of anemic patients attending follow-up clinics at the TASH. The study subjects included both genders, between 3 months to 68 years of age. Information was collected from the patients by a questionnaire designed to find out the relationship of hemoglobin variants with gender, age, family relation ship, etc. Blood films were prepared to look for morphological appearances of red cells and also malaria parasites. Out of 113 patients studied, only one was found to have malaria parasite on blood film. 90 % of the morphologies revealed variable size and shape of the red blood cells. The CBC revealed that the Hb of the patients ranged from 3.0 to 12.0 gm/dl and the MCV and MCH were 56.1-122.0 fl and 14.1-50.5pg respectively. The hemolysate that was subjected to agarose and Native-PAGE revealed that all the patients had HbA, HbF and $HbA_2$ and three patients (2.65 %) of the participants had sickle hemoglobin band. There were six patients (5.3 %) with thick $HbA_2$ band. It is clear from this study that there is a need to conduct this type of research on larger sample using sensitive methods, including DNA analysis to investigate the prevalence and distribution of Hemoglobinopathies in Ethiopia.

## 2. Introduction

Hemoglobin (Hb) is a red blood cell protein that transports oxygen from the lungs where oxygen tension is high to the tissue to meet the need of the cells for oxygen. It carries carbon dioxide from the tissues back to the lungs. To provide adequate oxygen transport, each RBC must enclose a high concentration of Hb. This concentration is close to the solubility limit of the molecule in physiologic solution. Therefore, even minor structural disturbance that alters isoelectric point, oxidation state, etc. can reduce solubility of Hb and compromise oxygen transport (Steinberg and Benz, 2000).

A molecule of Hb consists of four polypeptide chains with a small central cavity. Each polypeptide chain has a pocket on the external surface that contains heme moiety i.e. a complex of protoporphyrin IX and ferrous ($Fe^{2+}$) ion, positioned in a manner optimal for reversible binding of oxygen (Benz, 2005; Steinberg et al., 2000). The Hb tetramer opens and closes slightly as $O_2$ is taken up or released. Numerous non-covalent bonds are formed between the heme and the surrounding amino acid residues of globin subunits. An iron atom in the center of the porphyrin ring binds distal (E7) Histidine residue (Steinberg and Benz, 2000). Almost all the iron taken up by the reticulocytes is used for the synthesis of heme: the prosthetic group of Hb. Lack of iron limits the production of Hb and iron deficient erythropoiesis develops. The later is a common cause of anemia. Approximately one third of the world's population is believed to be iron deficient (Amha, 2005).

Different types of Hbs are produced during embryonic, fetal and adult life. Each consists of a tetramer of globin polypeptide chains (Benz, 2005). These polypeptides are a pair of α and α like globin chains and a pair of non α- chain that may be β, γ, δ and ε (Fairbanks and Klee, 1996).

In humans the first red blood cells containing embryonic hemoglobin appear at about 6 weeks of gestation (Benz, 2005). These are hemoglobin portland, hemoglobin Gower 1 and hemoglobin Gower 2 which are designated $\zeta_2\gamma_2$, $\zeta_2\varepsilon_2$, and $\alpha_2\varepsilon_2$ respectively (Fairbanks and Klee, 1996). The switch to nearly exclusive synthesis of adult Hb occurs at about 38 weeks of gestation (Benz, 2005).

The normal adult Hemoglobin is HbA ($\alpha_2$ $\beta_2$) that contains α-chain of 141 and β-chain of 146 amino acids with empirical chemical formula of $C_{2952}$ $H_{4664}$ $N_{812}$ $O_{832}$ $S_8$ $Fe_4$. Hemoglobin from a healthy individual is 96 % A, and 2.5-3 % $A_2$, and the latter is $\alpha_2\delta_2$. Fetal hemoglobin (HbF) predominates during fetal life but rapidly diminishes during the first year of life. The latter is $\alpha_2$ $\gamma_2$ and makes < 1 % of the total adult Hb (Fairbanks and Klee, 1996). HbF can be differentiated spectrophotometerically from HbA by the presence of a Tryptophan band i.e absorbance at 252nm, a consequence of an extra tryptophan at γ-130 (Nagel and Steinberg, 2001). HbF provides some protection to neonates and young children from dying of *plasmodium falciparum* malaria. HbF is more resistant to malarial hemoglobinase, hence red cell with this isomer of hemoglobin retards the growth of malarial parasites (Nagel and Steinberg, 2001). It was observed that HbF resists denaturation at alkaline pH. This is attributed to Threonine and Tryptophan at position 112 and 130 respectively in γ chain. In contrast to γ -globin, β -globin has Cysteine and Tyrosine residue at position 112 and 130 respectively i.e. residues that are ionized

at alkaline pH (Nagel and Steinberg, 2001). The δ-globin chains of $HbA_2$ differ from β-globin chain of HbA by ten amino acids out of 146. $HbA_2$ and HbA have identical Bohr effect, cooperativity, and response to 2,3-BPG, though the former has greater thermal stability. In vivo, it inhibits the polymerization of HbS. It is speculated that δ-22 Alanine and δ-87 Glutamine may be the important factors to resist HbS's aggregation (Nagel and Steinberg, 2001).

Obviously, the behavior of Hb is determined by its primary structure. In α chain, there are eight helical segments: A**,….,**H separated by short stretches of random coil. These non-helical segments permit folding of the polypeptide upon itself. The folding of the protein is responsible for tertiary structure of globin that places polar residues on the surface and a hydrophobic pocket of heme ring between helix E and F (Steinberg and Benz, 2000).

The two α-globins and two non α-globin chains fit together establishing quaternary structure with a molecular weight of about 68 KD (Steinberg and Benz, 2000). The amount and type of human Hb produced at any given age is determined primarily by the selective expression of the individual gene encoding each globin chain. This is the result of the sequential activation and inactivation of α- and non α- globin clusters. In human α like genes are on about 30 kb of DNA on the short arm of chromosomes 16 and the β - like genes are on about 70 kb of DNA on the terminal portion of the short arm of chromosome 11 (Steinberg and Benz, 2000). On the other hand ζ- and ε-genes are on the long arm of chromosome 16 and 11 respectively (figure 1).

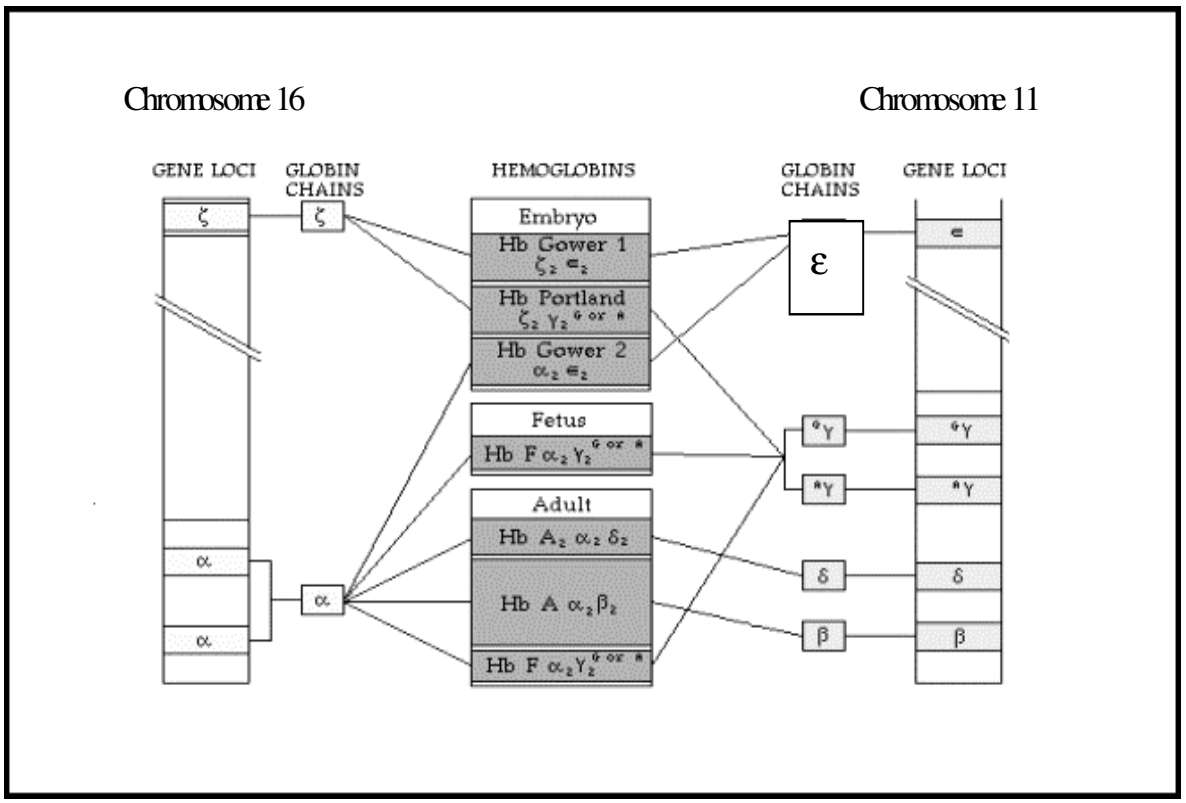


Figure 1. The globins gene loci on chromosomes 16 and 11. Taken from Barry S. https://www.medschool.lsuhsc.edu/Pathology/courses/Path201/ClassManual/Hemoglobinopathies.htm

Oxygen uptake and release can be studied by measuring $O_2$ content of samples spectrophotometerically. The data is plotted as $O_2$ saturation versus partial pressure of $O_2$ ($pO_2$) in the sample's environment. As figure 2 demonstrates, this curve is sigmoidal. Efficient oxygen delivery requires its uptake in the lungs and dissociation in the tissues. This function depends on the following factors: structure of the tetramer, temperature, pH, carbon dioxide, chloride and 2,3-BPG (Steinberg and Benz, 2000). This shape is the result of interaction among subunits of Hb and it is called heme-heme interaction or co-operativity (Steinberg and Benz, 2000).

Proton lowers hemoglobin's oxygen affinity leading to deoxygenation (Bohr effect). This arises from the stabilizing action of proton on deoxyhemoglobin, which binds proton more readily than

oxyhemoglobin i.e Hb has lower oxygen affinity at low pH, facilitating delivery of oxygen to tissues (Benz, 2005).

In addition to drop of pH, $CO_2$ and 2,3-BPG increase in the tissues. The later is due to increasing effect of glycolysis under less aerobic condition. As concentration of 2,3-BPG increases to equimolar concentration of Hb, it lowers oxygen affinity of the later. Specific amino acids involved in binding of 2,3-BPG are N-terminal valines, EF6/ $lys^{82}$ and H21/ $His^{143}$ of the β -chain (Steinberg and Benz, 2000). On the other hand, HbF does not bind 2,3-BPG, so it has higher affinity for oxygen. The primary structural basis of this effect is the replacement of $\beta^{143}$ Histidine, by Serine at phosphate binding site in the central cavity thus abolishing an important binding site (Nagel and Steinberg, 2001).

Carbonic anhydrase catalyzes the dissolution of $CO_2$ in blood generating $H^+$ and $HCO_3^-$. Carbon dioxide can also react with Hb generating carbamates because the unionized form of the α-amino group of Hb can react reversibly with $CO_2$. The bound carbamates form salt bridges that stabilize Tense-form (deoxy) of Hb conformation, hence, the binding of $CO_2$ lowers oxygen affinity. Temperature also affects oxygen binding to Hb. Increase in temperature shifts the oxygen dissociation curve to the right.

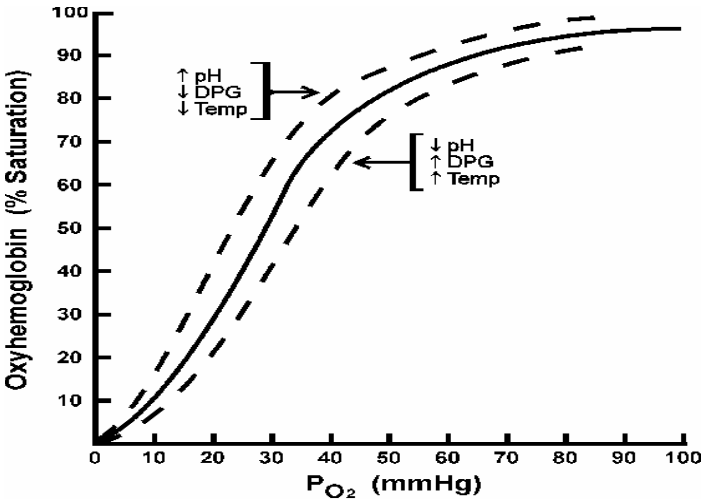


Figure 2. Oxygen dissociation curve of hemoglobin

Recently, It was found out that in the lungs Hb forms a covalent bond with nitric oxide establishing β Cysteine$^{93}$ S-nitroso group (Steinberg and Benz, 2000). HbA is able to transport nitric oxide, as S-nitrosohemoglobin, from the arterial to the venous blood. S-nitrosation significantly increases the oxygen affinity of the adult Hb with respect to native protein (Clementi et al., 2003) thus compromising the delivery of this vital molecule to tissues.

Structural defects of hemoglobin like sickle cell anemia, polycythemia, thalassemia, cyanosis, and other defects of erythrocytes lead to hemolytic disorders. To understand these erythrocyte disorders, we need detailed knowledge of the basic biochemistry, physiology, and molecular biology of the red cell membrane, enzymes, and hemoglobin.

Analysis of hemoglobin is important to identify congenital defects, which are called hemoglobinopathies and thalassemia. They are disorders affecting the production, the structure and function of Hb. These conditions are usually inherited and range in severity from clinically

asymptomatic laboratory abnormalities to death in utero (Benz, 2005). They are inherited diseases due to primary mutations affecting the globin genes. So far, nearly 1000 mutations that alter the expression, developmental regulation of individual globin genes or structure, were identified. However, most of these do not produce clinical diseases (Benz, 2000). Hemoglobinopathies may be divided into functionally distinctive groups. Some form polymers as in HbS, crystals as in HbC that give rise to hemolytic disease. The other is unstable hemoglobin generating Heinz bodies producing either intermittent or chronic hemolysis. Structural changes that increase the affinity of Hb for oxygen as in congenital familial erythrocytosis, lead to the rise of erythrocytes. Contrary to this, there are structural changes that decrease oxygen affinity for instance cyanosis. The last one is mutation that maintains Hb iron in the ferric state like in HbM (Lukens, 1999).

The physiologic regulator of red cell production is erythropoietin (EPO) released by renal peritubular cells. When Hb concentration drops below 10 to 12 gm/dl, plasma EPO level increases in proportion to the severity of the anemia. It induces RBC production in erythroid commited cell in the bone marrow. If this process is much lower than rate of destruction, it leads to anemia (Schnall et al., 2000). The later can be subdivided on the basis of red cell count and indices.

The number of physiologic factors that affect the normal red cell count and indices include age, gender, altitude, and pregnancy. (Adamson and Longo, 2005). The normal MCV is 80-100 fl. Values above and below this range define macrocytic and microcytic anemia respectively. The microcytic anemia have their origin in problems that cause deficient Hb synthesis such as in iron

deficiency anemia and thalassemia. On the other hand, significant macrocytic anemias (MCV>115 fl) usually result from folate and vitamin $B_{12}$ deficiency, since lack of these vitamins block DNA synthesis (Schnall et al., 2000).

The red cell distribution width (RDW) is a quantitative estimation of anisocytosis. The normal value is 13.4 ± 1.2%. The RDW may be useful when characterizing microcytic anemia, particularly distinguishing between iron deficiency anemia (high RDW, normal to low MCV) and uncomplicated heterozygous thalassemia (normal RDW, low MCV) (Perkins, 1999).

Sickle cell anemia and the thalessemias are the most important mutations that cause clinical morbidity (Benz, 2000). The thalassemia syndromes are a heterogeneous group of inherited anemia characterized by defects in the synthesis of one or more of the globin chain. This might result from Promoter region mutation, Splice junction mutation, RNA cleavage defect, chain termination mutation, etc. (Lukens, 1999).

Thalassemias have been encountered in virtually every ethnic group and geographic area. Like sickle cell anemia, thalassemia is most common in areas historically afflicted with endemic malaria (Forget, 2000). These genetic disorders confer protection against falciparum malaria. For example, HbA/S heterozygotes have a six-fold reduction in the risk of dying from severe falciparum malaria (White and Breman, 2005). Thalessemias are most common in the Mediterranean, equatorial or sub equatorial regions of Asia and Africa. The prevalence in this area is in the range of 2.5-15%. Some common thalassemia variants are HbE, Hb constant spring and Hb Lepore (Benz, 2000).

Inheritance of a single β-thalassemia allele results in a mild hypochromic microcytic anemia. The MCH (normal range 26-32 pg) is less than 26 pg and MCV is less than 75 fl. The smear shows varying types of cells**:** poikilocytes, ovalocytes, basophilic stippling and target cells (Schwartz and Benz, 1991).

Structural alteration of Hb molecule includes amino acid deletions, substitutions, addition, and fusion protein of two different genes (Lukens, 1999). Out of 634 Hb variants characterized by single amino acid substitution, 199 involve the α chain, 337 the β chain, 28 the δ chain, and 70 the γ chain (Lukens, 1999). Some examples are HbS (β6 Glu → Val), HbC (β6 Glu → Lys), HbE (β26 Glu → Lys), Hb Pyrgos (β83Gly→Asp), Hb Knossos (β27Ala→ Ser) and some examples from two amino acid substitutions are $HbS_{Travis}$ (β6 Glu →Val; β142 Ala → val) and $HbS_{Antilles}$ (β6 Glu → val; β23 Val→ Ile).

Malaria attacks the erythrocyte by entering into the blood stream. After invading an erythrocyte, the growing malaria parasite progressively consumes and degrades intracellular proteins, particularly Hb (White and Breman, 2005). The potentially toxic ligand i.e. heme is polymerized to biologically inert hemozoin, or malaria pigment. However, this process affects the RBC membrane and in turns its mobility. It exposes cryptic surface antigens, of parasite origin. The RBC becomes more irregular in shape, more antigenic and less deformable. This results in the sequestration of RBC's in vital organs where they interfere with microcirculatory flow and metabolism and shorten RBC survival. The accelerated RBC destruction will result in anemia (White and Breman, 2005).

Sickle shaped red cells were first reported by a Chicago physician, J.B. Herrick in 1910 after examining the blood of an anemic dental student from Grenada (reviewed by Steinberg, 2001). Sickle cell disease has its greatest frequency in Africa. In the North it is limited by the Sahara and the high lands of Ethiopia and spread southward to the Zambezi and Kunene rivers (http://www.tmcr.usuhs.mil/tmcr/chapter31/intro.htm). In 1925 the first case of sickle cell disease was reported in Africa. A 10-year-old boy was admitted to a hospital in Omduraman, Sudan with severe weakness. Microscopic study revealed sickle cell anemia (www.nslc.wustl.edu/sicklecell/biogeography.html). Later other confirmatory methods were developed. The abnormality of the Hb was confirmed in 1949 when Pauling and his colleagues reported that the electrophoretic mobility of HbS differ from that of normal HbA (Embury and Vichinsky, 2000). In 1957 Ingram discovered the physical basis of HbS to be the substitution of Valine for Glutamic acid in position six of the β- globin chain. In 1978 Kan and Dozy reported that the $\beta^s$-globin gene was in linkage disequilibrium with a polymorphic site located in its 3` flanking region that was identifiable by an HpaI restriction endonucleases cleavage site (Nagel and Steinberg, 2001).

HbS is freely soluble when fully oxygenated. However, if oxygen is removed, it polymerizes to crystals that are inflexible hence the cell sickles (Nelson, 1979). It generates sticky membranes that are abnormally adherent to the endothelium of small capillaries. These abnormalities provoke vasoocclusion of capillaries and premature RBC destruction. Hemolysis occurs because the abnormal erythrocytes are destroyed by the spleen (Benz, 2005).

There are 30 million sickle cell carriers in the world. In patients with no unique red cell morphology, red cell indices, and normal reticulocyte count, sickle forms are not seen on the peripheral blood smear (Embury and Vichinsky, 2000). For sickle cell disease some of the clinical symptoms are intermittent episodes of ischemic pain, hemolytic anemia and abnormal red cell morphology (Benz, 2005). The red cell life span is reduced to 15 to 20 days. Bone marrow analysis reveals excessive erythroid hyperplasia.

Epidemiological study showed 0.38-22.17 % in Egypt, 1.52-10 % in Sudan, 0.83-3.5 % in Algeria, 6 % in Tunisia and 0.005-4.4 % in Libya (Fawzi et al., 2003). In 2001, Steinberg showed the prevalence of sickle cell trait in east Africa to be 2-38 %. This African variation in prevalence is due to particularly geographic location of the population.

The other hemoglobinopathy is hereditary persistence of fetal hemoglobin (HPFH): continued synthesis of high level of HbF during adult life. HPFH shows ethnic differences. It was first observed in two healthy West African individuals who appeared to have sickle cell anemia with only HbS and fetal hemoglobin (Wood, 2001). HbF is elevated in hemolytic anemia and some bone marrow malignancies, such as meylodysplasia and also thalassemia (Fabry, 2001). Increased levels of HbF during stress results from premature maturation of erythroid proginator cells under the influence of high EPO (Wang and Lukens, 1999). Since synthesis of HbF peaks earlier than that of HbA so the proportion of fetal Hb is higher in immature cells compared with mature ones (Papayannopoulou and Abkowitz, 1991).

Many methods were developed to study hemoglobinopathies. Some of these are presented here. The first one is solubility test. Sickle Hb can be detected chemically because it is insoluble and precipitates in high molarity phosphate buffer. This is achieved by removal of oxygen with Sodium Dithionite solution in high salt (Steinberg, 2001). If HbS is present it gives a turbid appearance to the solution.

Hemoglobin electrophoresis is based on the different rates of migration of charged Hb molecules in an electric field. Many factors can influence this migration: pH, temperature, voltage, ionic strength of the buffer, and nature of supporting medium. In practice, the supporting medium may be cellulose acetate, starch gel, paper, agarose, or polyacrylamide (Kim and Schwartz, 1983). Agarose gel is usually used at concentrations of 1 % to 3 %, Polyacryamide gel electrophoresis (PAGE) is the most widely used method for protein analysis (Wilson and Walker, 2003). This method is based on the separation of protein according to size. It can be made with a content of 3% and 30%. It was observed gels of 15% polyacrylamide are useful for separating protein in the range 10 to 100 KD (Walker, 2003). The proteins in the gel are detected by staining. The most commonly used protein stain being Coomassie Brilliant blue R-250. Though its sensitivity is weak if a band contains, 0.1µg protein (Wilson and Walker, 2003).

Separation of hemoglobin by native electrophoresis is based on the relative charge of the tetramer and hence mutation that does not alter the charge may be “silent” and not detectable by electrophoresis. Those variants with similar charge will migrate together. Many hemoglobin variants overlap with each other. For example HbS overlaps with HbD, and HbG, $HbA_2$ overlaps with HbC, HbE, and HbO (Fabry, 2001).

The sequences of bands depend on the nature of amino acid substitution. For example, HbS has two more positive charges than HbA per hemoglobin tetramer, HbC has four more positive charges, and HbJ has two less positive charges under the same condition, than HbA, thus HbJ migrates more rapidly towards the anode than HbA. On the contrary HbS and HbC migrates more slowly than HbA (Adams and Steinberg, 1991).

Other methods include isoelectric focusing (IEF), high performance liquid chromatography (HPLC), and mass spectroscopy, genetic analysis by restriction endonucleases, specific oligonucleotide hybridization and DNA sequencing are also used (Steinberg and Junius, 2000).

## 3. Hypothesis

We suppose there are hemoglobinopathies particularly sickle cell anemia in Ethiopia.

## 4. Objective

### 4.1 General Objective

To identify the type of Hemoglobin abnormalities of anemic patients on follow up at the Tikur Anbessa Specialized Hospital.

### 4.2 Specific Objectives

1. To determine the hematological values of blood samples.
2. To study hemoglobin abnormalities by solubility test and electrophoresis.
3. To analyze the relationship between red cell morphology, red blood cell indices and Hb Variants

## 5. Ethical consideration

The project proposal was reviewed and approved by the ethical review committees of Addis Ababa University, Medical Faculty Research and Publication committee (FRPC II). Permission to conduct the study was also obtained from Internal Medicine and TASH as well. Informed written consents were obtained from the study subjects and also their guardians.

## 6. Materials and methods

### 6.1 Study design and period

The study is a cross sectional study that was conducted at the TASH during the period from December 2005 to July 2006.

### 6.2 Study area and study subjects

The study was conducted at Tikur Anbessa Specialized Hospital. The study subjects were anemic patients who had regular follow up in the hospital and their age ranged from 0.25 to 68 years of age (table 1). A physician had seen and selected each study subject before being included to the study. A Questionnaire was administered to find out the relationship of hemoglobinopathies with family relationship, age and gender. The collected samples were analyzed in the hematology laboratory of TASH, Core Lab., Biochemistry laboratory of Medical faculty.

Table 1. Study subjects by gender

| Study Subjects | Number | % of Total |
|---|---|---|
| Male | 63 | 55.75 |
| Female | 50 | 44.25 |
| Total | 113 | 100 |

## 6.3 Sample size

The sample size was calculated according to (Daniel, 1995) based on neighboring countries prevalence rate. Two most common hemoglobin variants in Africa are sickle cell anemia and thalassemia. The average prevalence rate of sickle cell anemia in the neighboring countries is 9.21 % and the prevalence of thalassemia in Africa is 15%. The average for both sickle cell anemia and thalassemia is 12.11 and the calculated sample size is 164**.**

$$N = (Z/d)^2 P(1-P)$$

$$= (1.96/0.05)^2 0.1211 (1-0.1211) = 164$$

Where N – number of samples Z – confidence interval (95 %)

P – proportion (12.11 %) d – the level of confidence is 5 %

Even though the calculated sample size was 164 this study included 113 subjects only, because it was not possible to get the calculated number within the given period.

- **Inclusion criteria**

All anemic subjects with a Hb level of < 13 gm/dl for males and < 12.1 gm/dl for females were included (Hughes-Jones, et al., 2004).

## 6.4 Hematology work up

Whole blood was collected by venous puncture and 1-2 milliliter blood was drawn from the vein using disposable syringe and collected into a test tube containing 2 mg EDTA. This sample was used for blood smear examination, complete blood counts, solubility test, and Hb electrophoresis.

### 6.4.1 Peripheral blood smear examination

Morphological study was done with thin blood smear stained with Wright stain for 3-5 minutes. The blood smear was examined under microscope (Olympus CX21) with objective (40x) for erythrocyte morphological abnormalities and with oil immersion at an objective of 100x for inclusion bodies and intraerythrocyte parasite such as plasmodium species.

### 6.4.2 Hemolysate preparation

Hemolysate preparation was carried out according to the Helena laboratories (http://www.helena.com). The anticoagulated blood was centrifuged in refrigerated centrifuge at 3500 RPM for 5 minutes. The RBC was washed three times with normal saline (0.9 % NaCl).

The washed cells were mixed with ¼ volume of Carbon tetrachloride and one volume of distilled water, vortexed and centrifuged at 3500 RPM for 10 minutes. The hemolysate, the upper red solution, was then separated by micropipette and stored in a refrigerator at - 70 $^{o}$C until electrophoresis was performed

### 6.4.3. Total Plasma protein

Total plasma protein was measured using Biuret method (Kingsley, 1942). Plasma and Biuret reagent containing Sodium hydroxide, Potassium tartarate, Copper sulphate and Potassium iodide were mixed. The absorbance of the mixture was measured at 546 nm against reagent blank with UV/Visible Spectrophotometer (Beckman, USA).

### 6.4.4 Solubility test

Solubility test was performed using a solution that contains dithionite ($Na_2S_2O_4$), saponin, and Potassium phosphate. Whole blood was mixed with 2 ml of the dithionite solution and left to stand for 5 min. The tube was then held at 2 cm from a white card with black lines to assess for transparency level.

### 6.4.5 Complete Blood Counts (CBC)

The complete blood count was done on automated blood cell counter (Sysmex KX-21N, Japan). It help to determine the presence or absence of anemia and other hematological abnormalities. It includes Hb, RBC counts, HCT, MCV, WBC, MCH, MCHC, RDW, and Platelets.

## 6.5 Electrophoresis

Electrophoresis was carried out on agarose gel and Native–polyacrylamide gel electrophoresis at pH 8.6 and 8.3 respectively.

### 6.5.1 Agarose gel Electrophoresis

Agarose gel electrophoresis was carried out on 1 % Agarose at pH 8.6 with Tris EDTA Borate buffer (Lepp and Bluestein, 1978). Agarose was dissolved in Tris EDTA Borate buffer and melted. The melted agarose was cooled and poured on horizontal gel cast on which there was a desired comb and left until solidified. The gel cast was placed in the elecetrophoresis chamber and the comb was carefully removed. Hemolysate that was diluted with electrophoresis buffer was applied to the agarose gel. The electrophoresis chamber was filled with electrophoresis buffer, sufficient to cover the gel. Each slot was loaded with 5µl hemolysate (total protein 1.4 µg/µl) and elecetrophoresis was carried out at 100V for 6 hours using Tris EDTA Borate buffer. After electrophoresis the gel was stained with Coomassie brilliant blue R 250. It was destained with aqueous solution of acetic acid and methanol and viewed.

### 6.5.2 Native-PAGE electrophoresis

The hemolysate was subjected to native PAGE electrophoresis (Robert and Prentis, http://www.msstata.edu/org/MAS/julyjournal/hamil.html). Electrophoresis was performed on 15 % running gel and 5 % stacking gel. The electrophoresis buffer used was Tris-glycine, pH 8.3.

Samples were prepared by mixing 5 µl hemolysate containing 1.4 µg/µl of Hb, with 100 µl of sample buffer. The sample buffer contains bromophenol blue, Tris buffer, pH 6.8, glycerol and distilled water. Each slot was loaded with 5 µl hemolysate and elecetrophoresis was carried out at 100 V and 20 mA for 24 hours with Mini protean II system (BIO-RAD, USA). The distinction between hemoglobin variant and normal Hb was made by running Hb control AFSC together with patients' hemolysate (Figure 3). The hemoglobin control AFSC contains 44% HbA, 25% HbF, 17% HbS and 14% HbC (Analytical control Systems, INC., USA). After electrophoresis the gel was stained with Coomassie brilliant blue R 250 for 2 hours and destained with solution containing 7% acetic acid and 5% methanol with consecutive washing and viewed.

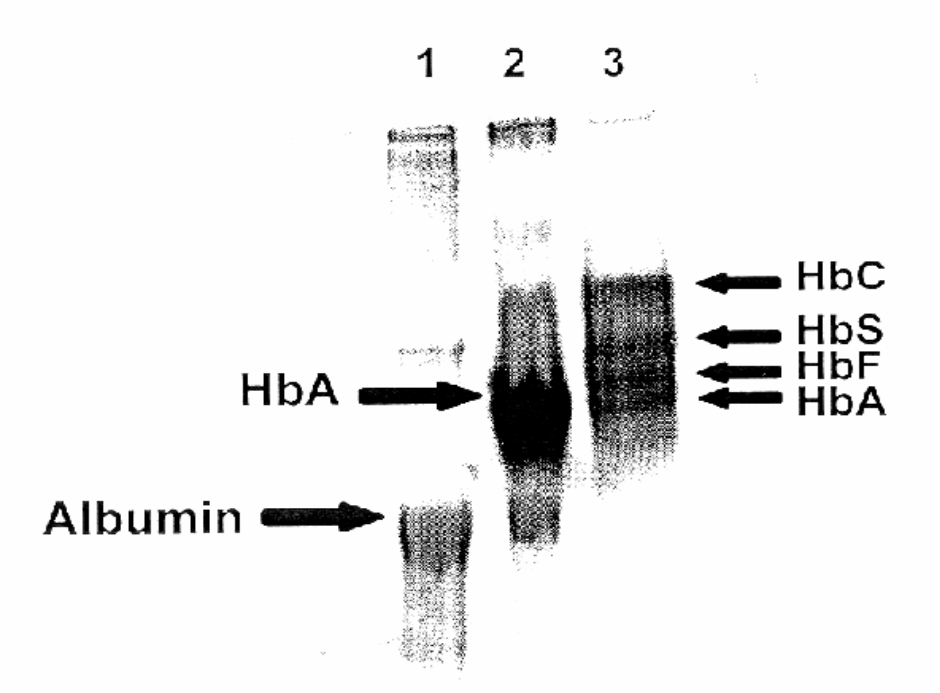


Figure 3. Results from native PAGE. Lane 3 loaded with hemoglobin standards. Lane 2 loaded with red blood cell extract from blood sample with hemoglobin A. Lane 1 loaded with serum from blood sample with albumin (Taken from Robert and Prentis)

## 7. Data analysis

Information obtained from questionnaire and laboratory analyses were entered into excel and STATA, and analyzed using Statistical package STATA software version 7.0.

## 8. Results

In this cross sectional study, a total of 113 anemic subjects participated. Out of the 113 subjects 63 (55.75 %) were males and 50 (44.25 %) were females (table 1). 50 % of the participants were below the age of 15 years (figure 4). The median and the mean age of the study participants were 12 and 19.7 respectively.

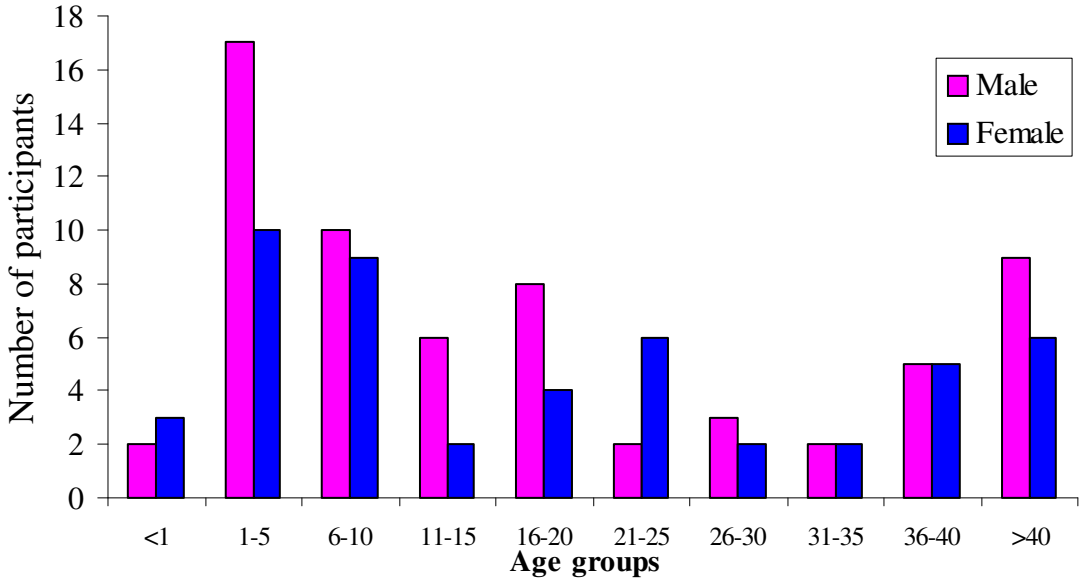


Figure 4. Age distribution of study participants

## 8.1 Morphological analysis

Peripheral blood smear revealed gross abnormality in the morphological appearance of the red blood cells as shown in (Figure 6). Anisopoikilocytosis was observed in 90% of the cases. Variations in shape include elliptocytes, anchantocytes, spur cells, teardrops, stomatocytes, etc.

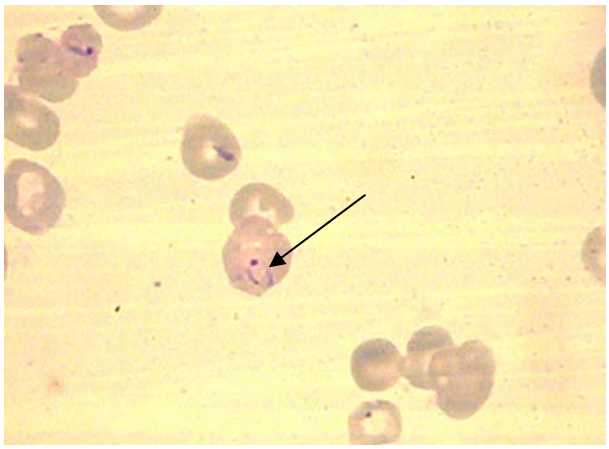

Figure 5. Morphology of erythrocyte of a male Patient aged 2 years and 2 months with malaria

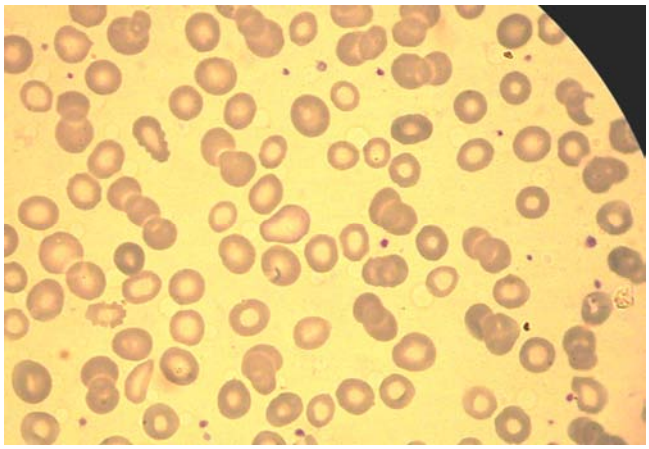

Figure 6. Morphology of erythrocytes of a female patients aged 6 years with variable size and shape and positive for solubility test

One patient had malaria parasite in the peripheral blood smear. He had double infections with *P. falciparum* and *P. vivax* (figure 5).

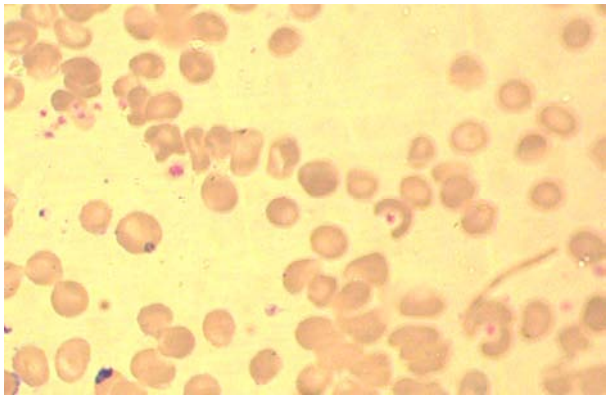

Figure 7. Morphology of erythrocytes of a patient with Sickle cell aged 23 months (male).

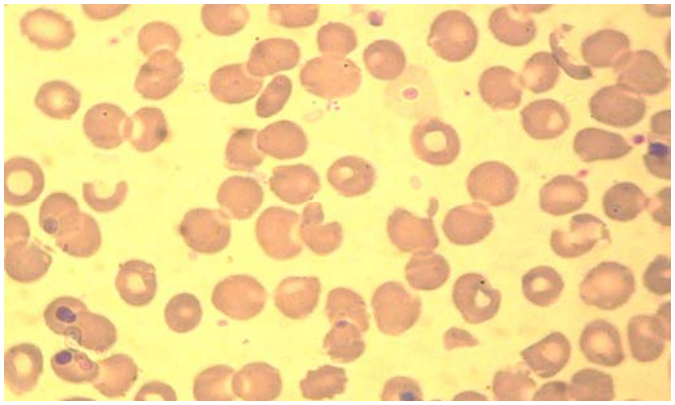

Figure 8. Morphology of erythrocytes of a patient with sickle cell, aged 17 years (male)

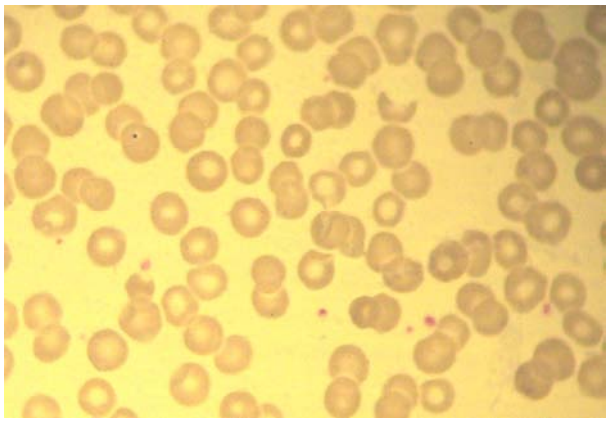

Figure 9. Morphology of erythrocytes of a 10 year old patient with Sickle cell.

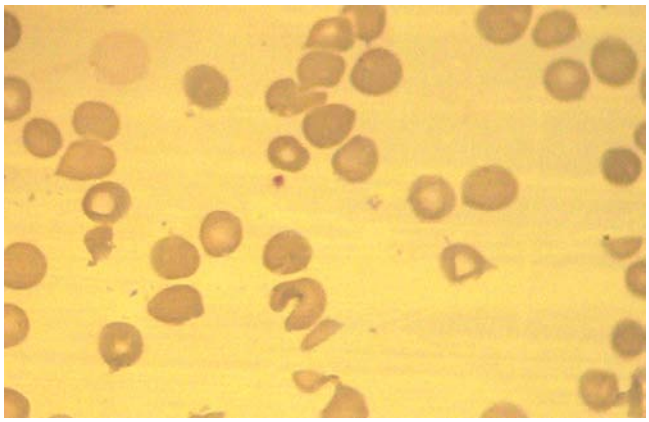

Figure 10. Morphology of erythrocytes of a patient aged 19 months with positive solubility test

## 8.2 Total Plasma Protein

Total Plasma protein is the measure of the total amount of protein in the blood. The normal value ranges from 6.6 - 8.7gm/dl for children from 3 years old and adults and the range for infants is from 4.6 - 7.0 gm/dl (Weichselbaum, 1946). Total protein in the plasma of the 113 patients ranged from 3.02–12.6 gm/dl.

## 8.3 Complete Blood counts

Mild to severe degree of anemia was observed in all the subjects based on the red blood cells counts (figure 11-16). The Hb concentration varied from 3.0 - 12.0 gm/dl for females and 3.4 - 13.0 for males. There were 42 patients (37.2 %) that had MCV < 80 fl and 12 patients (10.6 %) had MCV > 100 fl. 59 patients (52.2 %) had normal MCV value. 35 patients (31 %) had MCH < 26 pg. There were 20 patients (17.7 %) that had MCH > 32 pg. There were 12 patients (10.62%) that had MCHC > 36 gm/dl and 40 patients (35.4 %) that had MCHC < 32 gm/dl. The rest 61% had normal ranges between 32 and 36 gm/dl. Out of the 113 patients, Red cell width (RDW) was

determined in 28 patients. RDW distribution ranged from 12.5 - 25.8 %. Of these pateints, nine of them had RWD below 14.6 % that is within the normal range. The 19 had shown higher value.

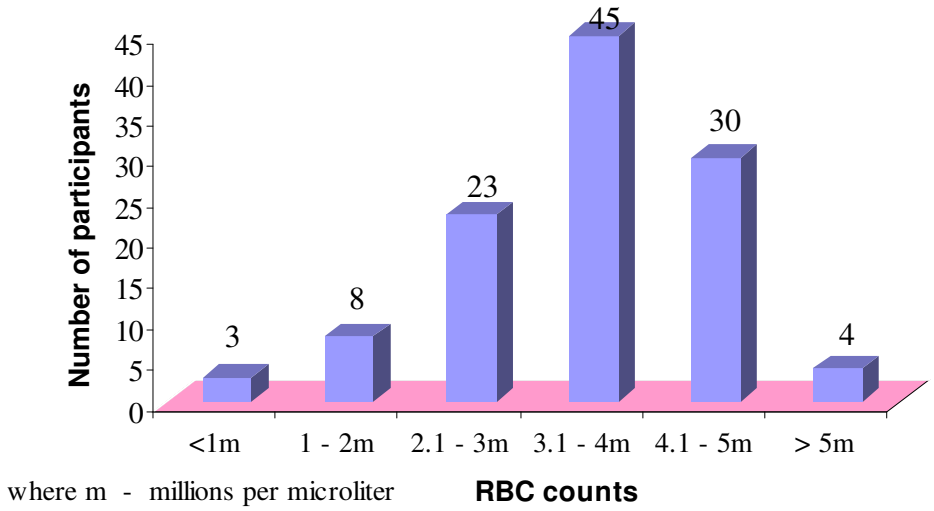


Figure 11. RBC distributions among study participants

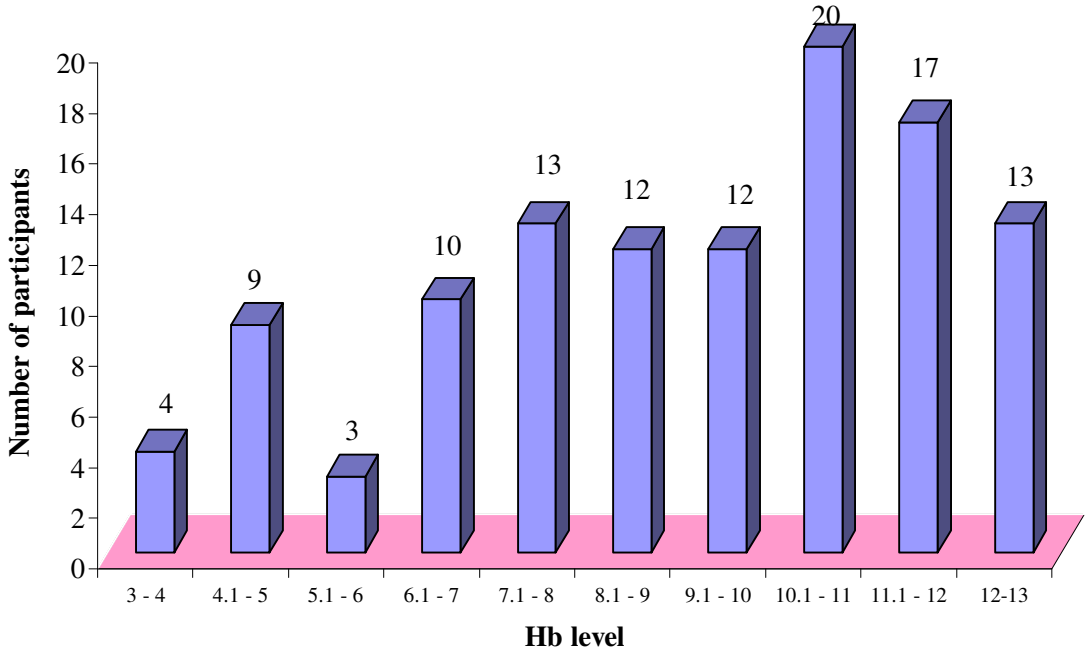


Figure 12. Hb (gm/dl) of the study participants

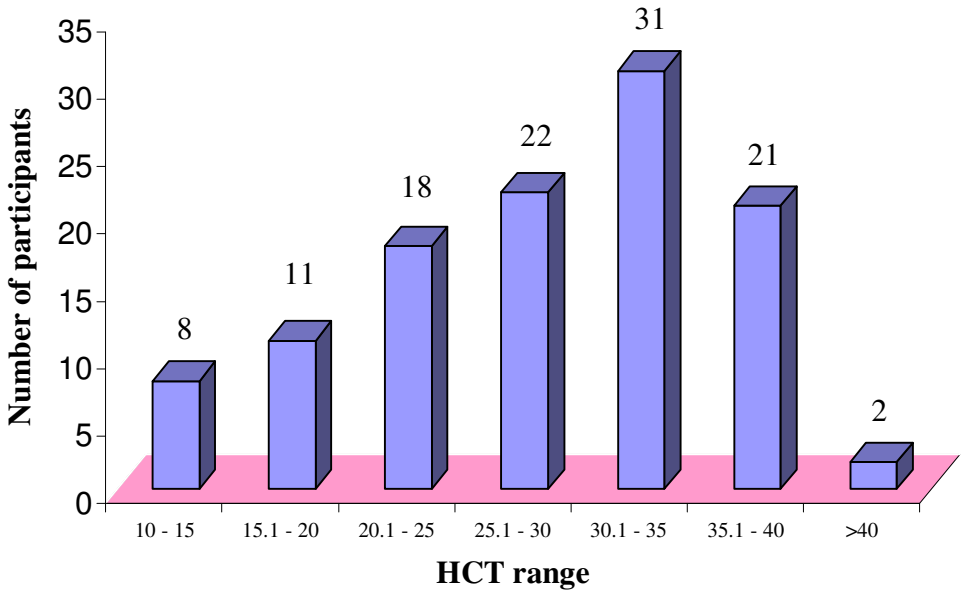


Figure 13. Hematocrit of study participants in % of sample volume

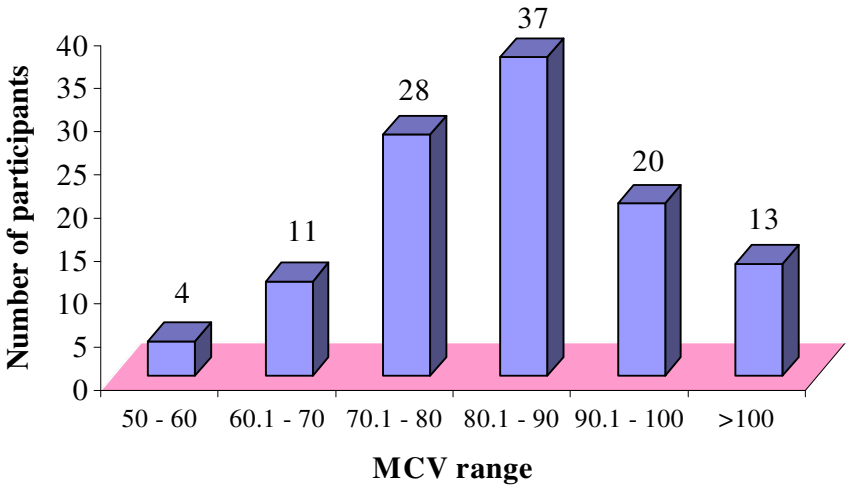


Figure 14. MCV (in fl) of study participants

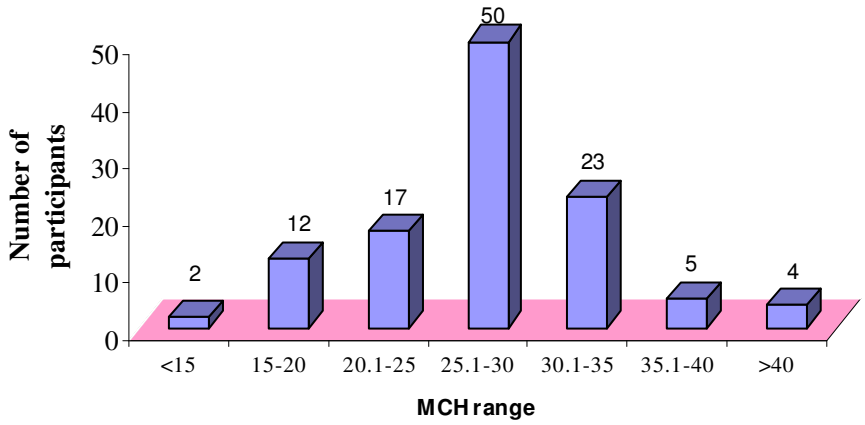


Figure 15. MCH (in pg) of study participants

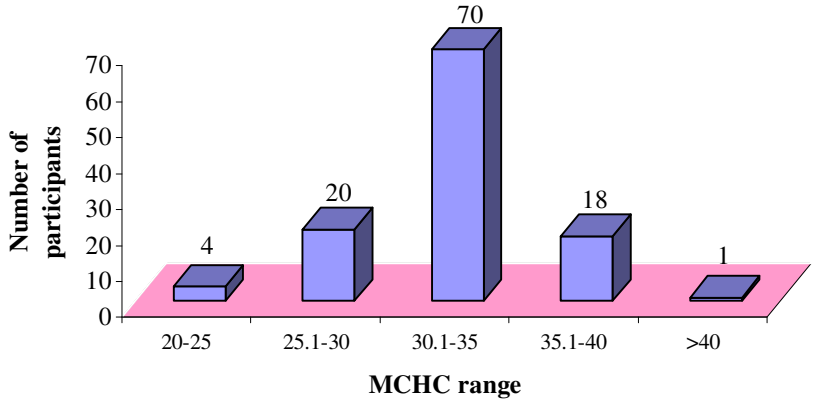


Figure 16. MCHC (in gm/dl) of study participants

## 8.4 Solubility Test

There were 5 samples that were positive for solubility tests, the rest were negative. Among these one was 6 year old child where as the others were below 2 years of age. These patients showed increased WBC count**.** There were also patients who were negative for solubility test and had no sickle cell on morphology (figure 7, 8 and 9) but proved to have sickle cell by electrophoresis.

## 8.5 Electrophoresis

### 8.5.1 Agarose gel electrophoresis

The agarose gel electrophoresis revealed HbA clearly but the other Hb types were not properly resolved (figure 17).

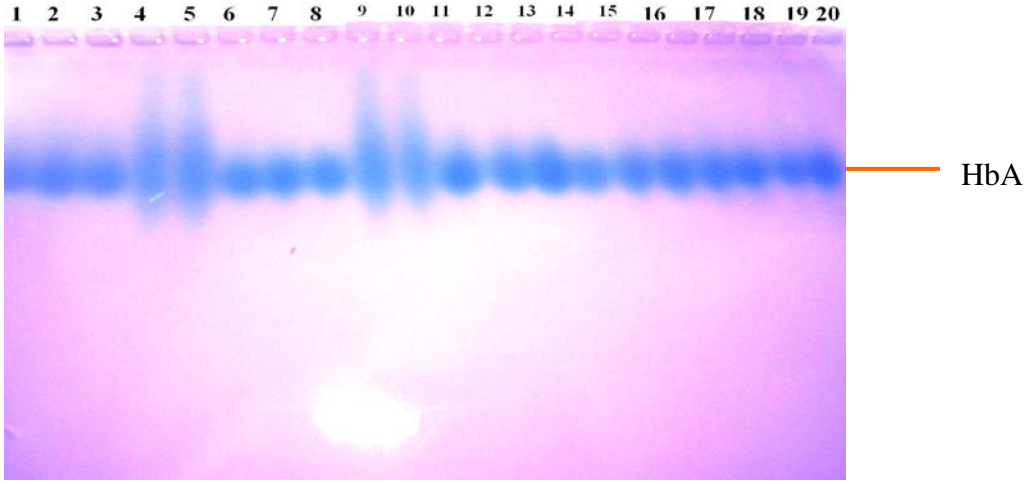


Figure 17. Agarose gel electrophoresis of hemoglobin

**Legend**:- Lane -1, 2, 3, 6, 7, 8, 11, 12, 13, 14, 15, 16, 17, 18, 19,20 - anemic patient hemolysate
Lane - 4, 5, 9 and 10 - Hb control

### 8.5.2 Native PAGE

Native PAGE electrophoresis showed, HbA with two thin bands, $HbA_2$ and HbF and very faint band that is unknown (figure 18). In all the subjects, all the four bands were observed. The difference lies on the thickness of the band. The thickness of the band can be attributed to the difference in their relative abundance. The control loaded on to gels along with samples did not show HbC. The sequence of the band is A, F, S, and C**:** A being the furthest, from the point of application (cathode).

Three anemic subjects aged 23 months (lane 3 and 4 figure 18), 10 years (lane 6 and 7 figure 18), and 17 years (lane 8 and 9 figure 18) with HbS were discovered by Native PAGE electrophoresis. These patients had sickle cell trait since their blood samples gave HbS band (Figure 18). These patients have HbA with normal intensity where as the HbS was thin band that showed the patients being recessive for the trait. The third patient who is male aged 17 years in particular had very faint HbS band.

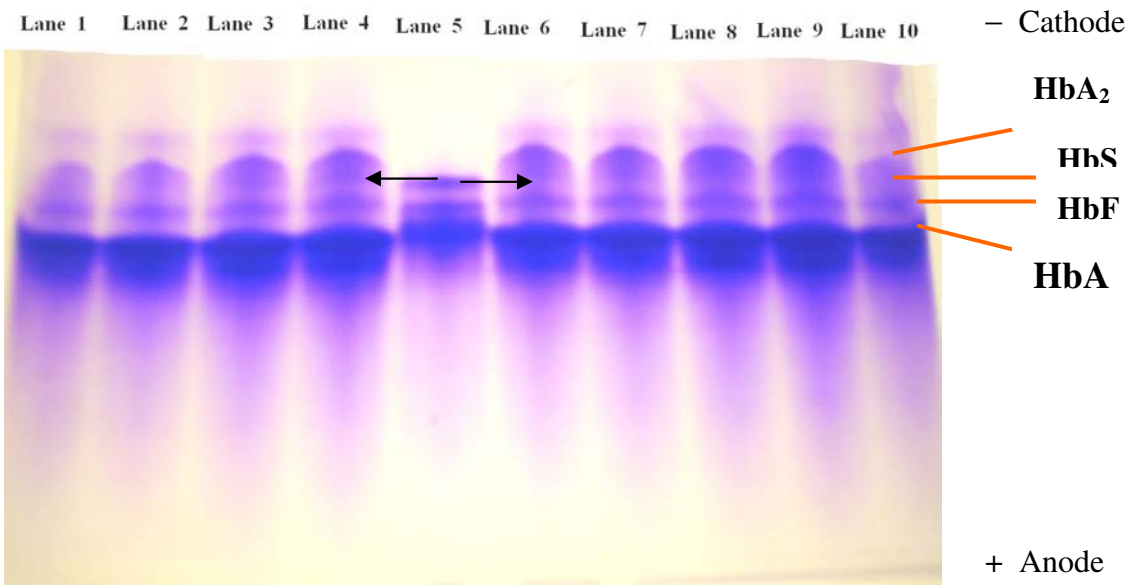


Figure 18. Native PAGE hemoglobin electrophoresis. There was HbS on the gel in between HbF and $HbA_2$.

**Legend:**

Lane 1 and 2 - male, aged 19 years
Lane 3 and 4 -male, aged 23 months with sickle cell
Lane 5 - hemoglobin control
Lane 6 & 7 - male, aged 10 with sickle cell
Lane 8 & 9 - male, aged 17 with sickle cell
Lane 10- female, aged 9 years with anemia

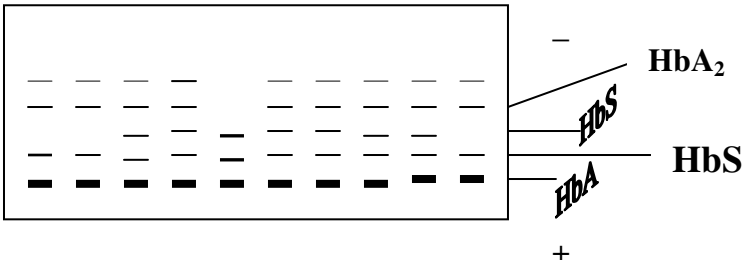


Figure 19. A Schematic representation of figure 18

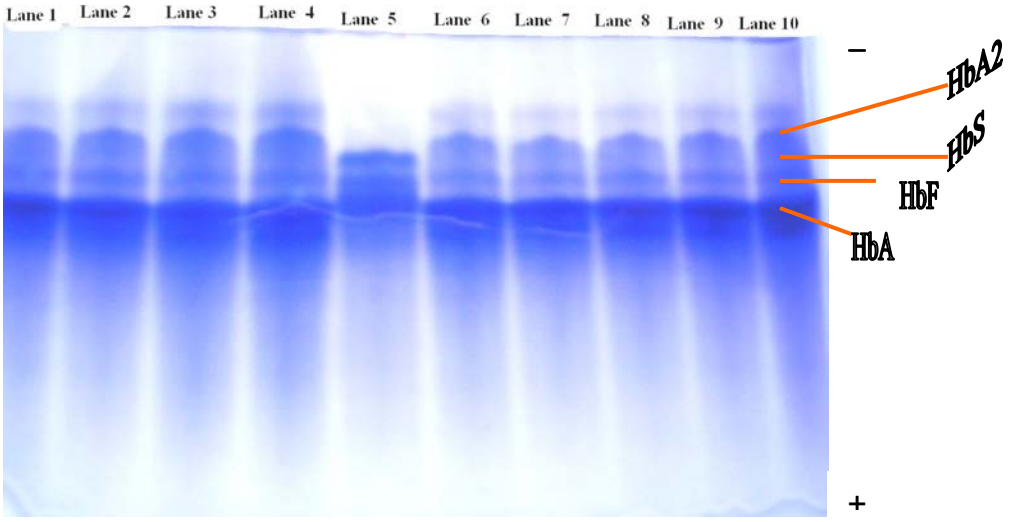


Figure 20. Result of Native PAGE hemoglobin electrophoresis.

**Legend:**

Lane 1 & 2 – Female aged 8 years with anemia
Lane 3 & 4 – female, aged 27 years with MBA
Lane 5 - hemoglobin control
Lane 6 & 7 – female, aged 23 years with MBA
Lane 8 & 9 – female, aged 29 years
Lane 10 – male, aged 1.45 years with anemia

There were six patients with thick $HbA_2$ band. Among these, two of them (27 years old female, lane 3 & 4 figure 20) and 28 years old females had megaloblastic anemia. The others were 23 years old female (lane 6 & 7 figure 22) and 19 years old male (lane 6 and 7 figure 21) with anemia. The last were children aged 17 months old boy and 6 years old female. These two children had higher WBC counts, 12000 and 11500 per µl respectively.

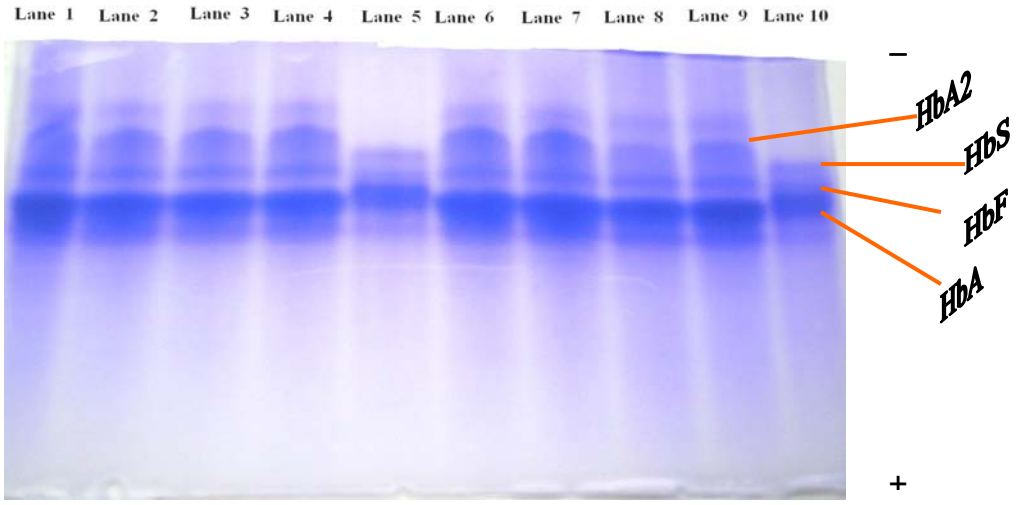


Figure 21. Result of Native PAGE hemoglobin electrophoresis.

**Legend:**

Lane 1 & 2 – female, aged 45 years with MBA
Lane 3 & 4 – female, aged 17 years
Lane 5 – hemoglobin control
Lane 6 & 7- male, aged 19 years
Lane 8 & 9 -male, aged 26 years
Lane 10 – Hb control

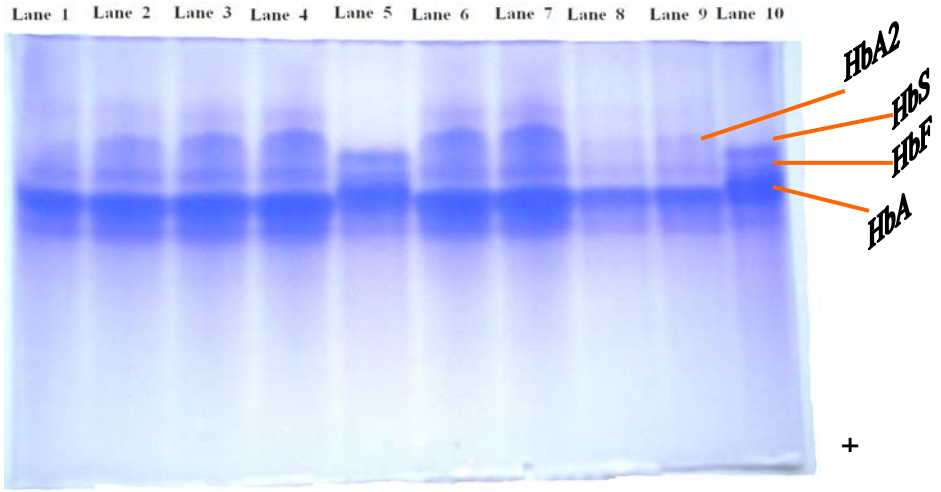


Figure 22. Result of Native PAGE hemoglobin electrophoresis.

**Legend:**

Lane 1 & 2 – male, aged 21 years
Lane 3 & 4 – female, aged 25 years
Lane 5 - Hb control
Lane 6 & 7 – female aged 23 years with thick $HbA_2$
Lane 8 & 9 – male, aged 39 years
Lane 10 – Hb control

Table 2. Result of patients that had sickle cell and bold $A_2$

| Patient code | Microscopy | Solubility test | Electrophoresis | | | | |
|---|---|---|---|---|---|---|---|
| | | | HbA | $HbA_2$ | HbF | HbS | Ub |
| 55 | Hypochromic, variable size | - ve | 1 | 1 | 1 | 1 | 1 |
| 92 | Normochromic and some are Hypochromic, variable size. | - ve | 1 | 1 | 1 | 1 | 1 |
| 97 | Hypochromic, variable size and shape | - ve | 1 | 1 | 1 | 1 | 1 |
| 7 * | Anisocytosis, hypochromic | - ve | 1 | 1 | 1 | 0 | 1 |
| 22 * | Variable size, normochromic, there are cells that are also hypochromic | - ve | 1 | 1 | 1 | 0 | 1 |
| 38 * | Hypochromic, variable size and shape | + ve | 1 | 1 | 1 | 0 | 1 |
| 45 * | Elliptocytosis, spherocytes, small and large RBC, hypochromic | +ve | 1 | 1 | 1 | 0 | 1 |
| 102* | Hypochromic, variable size and shape (spherocytes) | - ve | 1 | 1 | 1 | 0 | 1 |

* Patients that had bold $A_2$ Ub - unknown band 1 - presence of band
0 – absence of band

## 8. Discussion

Red cell morphological abnormalities were seen in 90% of the patients. There were variable shape and size in their red cell morphologies. Those patients with high MCV (macrocytosis) had spherocytes. There were three subjects that had sickle cell trait (see below). All of them had no sickle cell on the peripheral blood smear. This coincides with Embury and Vichinsky (2000) studies that reported sickle cell trait is benign and sickle cell forms are not seen on the peripheral blood smear.

The solubility test helps us to identify whether there is sickle cell Hb. Sickle Hb is insoluble and precipitates in high molarity phosphate buffer at neutral pH when it loses oxygen by Sodium Dithionite (Steinberg and Junius, 2000). Five patients had positive solubility test. However, electrophoresis analysis was negative for all of them. All the five patients had higher serum protein as well as WBC counts. These patients may have other Hb variants that show positive test for example Hemoglobin $C_{Harlem}$, $C_{ziquinchor}$, and $S_{Travis}$ give positive result (Kim and Schwartz, 1983). Like wise, the presence of Heinz bodies, and blood protein disorders lead to precipitation of plasma proteins (http://www.labcorp.com/datasets/labcorp/html). Other reports showed that erythrocytosis, hyperglobulinemia, extreme leukocytosis or hyperlipidemia might cause false positive results (http://www.biotrondiagnostics.com/pdf/sickle%20cell%20uni-test.pdf). Two of them showed thick band for $HbA_2$ on electrophoresis. There were three (2.65%) patients with sickle Hb that was proved by electrophoresis but solubility test didn't confirm the presence of sickle cell trait. Since the HbS band is faint the individuals are heterozygotes and the amount of protein is simply too small to precipitate. Solubility test fails to detect the low concentration of HbS that might be present in some varieties of HbS-$\beta^+$ thalassemia or sickle cell trait with $\alpha$-thalassemia (Steinberg, 2000).

Proteins are important constituent of all cells and tissues. They are continuously broken down to amino acids that can be used to make new proteins like hemoglobin, hormones, enzymes and other compounds needed by the body. Total protein determination has many uses: - among these; it helps to diagnose rare blood disease such as plasma cell dyscrasias, and lymphomas and also liver disease and malnutrition etc. Among the 19 babies that were below 3 years old only one had < 4.6gm/dl, 11 of them had > 7 gm/dl total proteins and seven of them had normal range

(4.6 – 7 gm/dl). For those patients that are > 3 years of age, 28 of them had lower values i.e. < 6.6 gm/dl and 19 had higher value i.e. > 8.7 gm/dl. The rest (47) of them had normal value (6.6 – 8.7 gm/dl). Those patients that had lower values than normal might be due to malnutrition, malabsorption, over hydration, or hemodilution, etc. and those that had higher value might have severe dehydration, liver cell damage, chronic infection, plasma cell dyscrasias, etc (Silverman and Christenson, 1996).

Marked degree of hypochromia was observed in many of the patients. There were 12 patients (10.6 %) that had >100 fl MCV which may be due to folate and/or vitamin $B_{12}$ deficiency, or these patients may have been on drugs such as hydroxyurea that interferes with DNA metabolism, hence elevated MCV. The other reason might be due to RBC agglutination such as cold agglutinins that could falsely elevate MCV. Macrocytosis increases with increasing reticulocytosis since reticulocytes are polychromatophilic macrocytes, and also agglutination of RBC in the presence of cold agglutinins may lead to the false elevation of the MCV because aggregates may be sensed as large RBC (Berliner and Duffy, 1991). There were 42 patients (37.2 %) that had MCV < 80 fl who might have anemia of chronic disease or iron deficiency anemia. In order to identify iron deficiency anemia RDW was used. The RDW of RBC shows heterogeneity in size of RBC. Higher value shows greater variability in size (Schnall, et al., 2000). According to Lee, (1999) the normal value for RDW is 13.4 ± 1.2 %. In iron deficiency, the value is 16.3 ± 1.8 %. Of the 42 patients, RDW was determined in four only. Out of these one patient 23 months of age had RDW 17.7 %, low MCV (78.5fl) and, low MCH (24.5 pg) and were observed to be positive for HbS on electrophoresis. There were two other male patients that had sickle cell band on electrophoresis and the age of the patients were 10 and 17 years. One

of them (17 years) had 87.2 fl MCV and the RDW was not determined. The 10-year-old boy had an MCV 88 fl and RDW is 16.6 %. The red blood cell indices in these two patients are normal. This coincides with Steinberg (2000) that reported neither hematological indices measured by electronic cell counter nor peripheral blood film reviews are useful for diagnosis because both are normal in sickle cell trait.

Among the 28 patients that had RDW determination, it was normal in nine of them and 19 of them had higher RDW that may indicate iron deficiency anemia and/or megaloblastic anemia. An increased RDW value is an early and pronounced finding in iron deficiency and most megaloblastic anemia (Lee, 1999). The MCH and MCHC also showed defect in Hb synthesis, which ranged from 14.5 – 50.5 pg and 21.8 – 49.5 gm/dl respectively. There were 35 patients (30.97%) that had MCH < 26 pg and 40 patients (35.4%) had MCHC < 32 gm/dl that might indicate iron deficiency anemia. There were 20 patients (17.7 %) that had MCH > 32 pg and 12 patients (10.62 %) that had MCHC > 36 gm/dl.

The agarose gel didn't show the other Hb types this is in contrast to Ahmed et al., (2004) who observed 3 bands on the agarose gel but they used different staining chemicals. Six patients had thicker band of $HbA_2$ on electrophoresis. Among these, two females aged 27 and 28 years, had megaloblastic anemia as indicated by clinical diagnosis. The reason for the increased concentration of $HbA_2$ might be the megaloblastic anemia. As Barry S. pointed out, $HbA_2$ is increased in megaloblastic anemia and β-thalassemia (http://www.medschool.lsuhsc.edu/Hemoglobinopathies.htm). Perhaps the high $HbA_2$ of megaloblastic anemia is a result of more hemoglobin synthesis occurring in less mature erythroid precursors (Nagel and Steinberg, 2001). The other two patients that had bold $HbA_2$ band, aged 6

years (female) and 1 year and 5 month (male), were positive for solubility test and had higher WBC count 12000 and 11500 /μl respectively. They had also higher total serum protein (7.03 and 7.88 gm/dl), which might be an indication of chronic inflammation or infection. They became positive for solubility test because of the higher WBC counts and higher serum protein that lead to precipitation. In the last two patients aged 19 (male) and 23 years (female), the thick $HbA_2$ band might be drug induced. Some disease states that raise $HbA_2$ are sickle cell anemia, α-thalassemia, HIV, malaria, unstable hemoglobin, hereditary spherocytosis, congenital dyserythropoietic anemia and others (Nagel and Steinberg, 2001). Incidentally, a study conducted at high altitude in North Gonder, showed that there was no elevated $HbA_2$ levels in the study subjects (Beall et al., 2002).

## 9. Conclusion and Recommendations

Hereditary disorders that result in structurally abnormal Hb or insufficient quantity of Hb are the most common human genetic diseases. It is clear from this study that laboratory data such as microscopic examination for morphology, solubility test, Hb electrophoresis, etc. should be done and analyzed before drawing any conclusion about a patient. This work indicates that there may be low frequency (<1%) of sickle cell trait in Ethiopia. But there was no sickle cell anemia (homozygous) in the study subjects because the HbS band is thin (heterozygotes) as a result of this the solubility test is negative for the HbS patients. In the future, it may be necessary to conduct similar study on a larger sample using special investigation such as DNA restriction studies of reticulocytes. Further more, there is a need to measure the level of hematinics in patients with anemia moreover, the determination of serum iron studies and % transferin saturation, iron binding capacity etc. may also be important. These laboratory data together with molecular analysis studies may give a clearer picture about hemoglobin abnormalities including Hemoglobinopathies in Ethiopia.

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## Annex I Questionnaire

1. Introduction

1.1 Full name of the participant_____

1.2 Subject identification number_____

1.3 Date of interview_____

1.4 Place of interview_____

1. Age (in year)_____
2. Gender 1. Male ☐ 2. Female ☐
3. Nationality _____
4. Address _____

5. History of other diseases

6.1 infections (skin or other) 1. Yes ☐ 2. No ☐

6.3 Other diseases _____

6. History of Malaria/ Does the person has malaria

1. Yes ☐ 2. No. ☐

7. Does his/her family have history of anemia?

8.1 Anemia 1.Yes ☐ 2. No ☐ 3. Unknown ☐

8.2 Others specify _____

## Annex II Consent Form

Code number_____

Name of study subject_____

I have been informed about a study plans that deals with analysis of participants Hb. For this blood needs to be taken from me. The aims of the study and the possible risks, including mild pain were explained to me.

I am also informed that all the information contained with in the questionnaire is to be kept confidential. More over, I have also been well informed of my right to keep hold of information, decline to cooperate and make myself withdraw from the study.

It is therefore with full understanding of the situation that I gave the informed consent voluntarily to the researcher to use the blood taken from me for the investigation. More over, I have had the opportunity to ask questions about it and received clarification to my satisfaction. I have also been informed that the nature of the questionnaire is private.

Signature _____ Signature _____

(Participant) (Investigator)

Date _____

**Annex III Consent form (Amharic version)**

# የስምምነት ቅጽ

**የሚስጥር ቁጥር----------**

**የተሳታፊው ሙሉ ስም----------**

**እኔ ስሜ ከላይ የተጠቀሰው ውል ተቀባይ በደም ላይ (Hb Profile) ሊደረግ ለታሰበው ጥናት መረጃ አግኝቻለሁ፡፡ ለዚህ ይረዳ ዘንድ ከእኔ እጅ ላይ ደም መውሰድ እንደሚፈለግ ተረድቻለሁ፡፡ ስለጥናቱ አላማ፤ እንዲሁም ናሙና ሲወሰድ በእኔ ላይ መጠነኛ የህመም ስሜት ሊያስከትል እንደሚችል ከውል ሰጪው ገለጻ ተረድቻለሁ፡፡**

**በተጨማሪም መጠይቁ ውስጥ በተካተቱት ጥቄዎች መሰረት የምሰጣቸው መረጃዎች በጠቅላላ በሚስጥር እንደሚጠበቁ ተገልጾልኛል፡፡ እንዲሁም እኔን በተመለከተ የምጠየቀውን መረጃ ያለመስጠት፤ በጥናቱ ያለመተባበርና ከጥናቱ በማናቸውም ጊዜ ራሴን የማግለል መብቴ የተጠበቀ መሆኑ ተገልጾልኛል፡፡**

**ስለዚህ ለውል ሰጪው መረጃ እና የስምምነት ቃሌን የሰጠሁት በአጠቃለይ ሁኔታውን በመረዳትና በፍጹም ፈቃደኝነት ነው፡፡ ከኔ የሚወሰደው ናሙና ለምርምር እንደሚውል ተረድቻለሁ፡፡ በተጨማሪም ጥያቄ ለመጠየቅ ተፈቅዶልኝ ለማወቅ የፈለኩትን ያህል ማብራሪያ አግኝቻለሁ፡፡**

**የውል ተቀባይ ፊርማ ----------  የውል ሰጪ ፊርማ----------**

**ቀን----------**

## Annex IV CBC Data

| Patient Code | Age (Year) | Sex | WBC per µl | RBC per µl | Hb gm/dl | HCT % | MCV fl | MCH pg | MCHC gm/dl | PLT | RDW_ SD (fl) | RDW_ CV (%) | Total prot-ein (g/dl) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 35 | M | 13400 | 1380000 | 4.3 | 12.7 | 92.0 | 31.2 | 33.9 | 224000 | 55.0 | 15.9 | 7.08 |
| 2 | 45 | F | 3700 | 2120000 | 6.3 | 18.9 | 89.2 | 29.7 | 33.3 | 27000 | 53.5 | 16.3 | 8.00 |
| 3 | 17 | F | 10900 | 3360000 | 11.5 | 30.8 | 91.7 | 34.2 | 37.3 | 254000 | 50.4 | 16.3 | 3.16 |
| 4 | 16 | F | 9900 | 3980000 | 11.8 | 36.1 | 90.7 | 29.7 | 32.7 | 250000 | | | 7.27 |
| 5 | 36 | F | 7600 | 4210000 | 10.4 | 34.0 | 80.8 | 27.1 | 33.5 | 211000 | | | 7.09 |
| 6 | 30 | M | 11600 | 2570000 | 4.6 | 16.1 | 62.6 | 17.9 | 28.6 | 64000 | | | 8.51 |
| 7 | 19 | M | 1800 | 2800000 | 8.5 | 24.4 | 117.3 | 40.9 | 34.8 | 8000 | | | 7.84 |
| 8 | 40 | M | 7700 | 3700000 | 10.4 | 31.3 | 84.6 | 28.1 | 33.2 | 178000 | | | 5.66 |
| 9 | 13 | F | 12300 | 4340000 | 10.6 | 35.2 | 81.1 | 26.7 | 33.0 | 23000 | | | 7.41 |
| 10 | 40 | F | 3000 | 2650000 | 7.8 | 22.6 | 85.3 | 29.4 | 34.5 | 53000 | | | 5.03 |
| 11 | 35 | F | 2300 | 3380000 | 10.4 | 30.2 | 89.3 | 30.8 | 34.4 | 68000 | | | 5.50 |
| 12 | 63 | M | 191700 | 2360000 | 7.3 | 21.5 | 91.1 | 30.9 | 34.0 | 122000 | | | 7.52 |
| 13 | 17 | M | 5700 | 4210000 | 12.6 | 38.0 | 90.3 | 30.0 | 33.2 | 449000 | | | 6.18 |
| 14 | 26 | M | 1800 | 1550000 | 6.5 | 18.5 | 119.4 | 41.9 | 35.1 | 54000 | | | 7.58 |
| 15 | 21 | M | 5600 | 3180000 | 9.8 | 29.8 | 93.7 | 30.8 | 32.9 | 46000 | | | 5.97 |

| Patient code | Age (year) | Sex | WBC per µl | RBC per µl | Hb gm/dl | HCT % | MCV fl | MCH pg | MCHC gm/dl | PLT | RDW_ SD (fl) | RDW_ CV (%) | Total protein (g/dl) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 16 | 43 | M | 5200 | 4280000 | 12.2 | 38.1 | 89.0 | 29.4 | 33.1 | 107000 | | | 7.80 |
| 17 | 6 | M | 7900 | 3440000 | 8.6 | 27.9 | 81.1 | 25.0 | 30.8 | 12000 | | | 7.67 |
| 18 | 25 | F | 4900 | 3610000 | 10.5 | 33.0 | 91.4 | 29.1 | 31.8 | 298000 | | | 10.29 |
| 19 | 21 | M | 1300 | 2270000 | 6.5 | 19.9 | 87.7 | 28.6 | 32.7 | 13000 | | | 7.65 |
| 20 | 68 | M | 12900 | 4580000 | 12.9 | 39.1 | 85.3 | 28.2 | 33.0 | 773000 | 49.0 | 14.3 | 9.50 |
| 21 | 39 | M | 3700 | 2760000 | 5.6 | 19.2 | 69.6 | 20.3 | 29.2 | 151000 | | | 7.15 |
| 22 | 23 | F | 5700 | 4260000 | 11.9 | 30.9 | 72.5 | 28.4 | 39.2 | 162000 | | | 7.63 |
| 23 | 40 | M | 8900 | 3890000 | 9.8 | 32.3 | 83.0 | 25.2 | 30.3 | 176000 | 50.8 | 16.6 | 8.42 |
| 24 | 9 | F | 26600 | 2010000 | 6.8 | 20.5 | 102.0 | 33.8 | 33.2 | 65000 | | | 9.81 |
| 25 | 39 | M | 3700 | 2810000 | 4.3 | 16.2 | 57.7 | 15.3 | 26.5 | 260000 | | | 9.71 |
| 26 | 11.5 | M | 6800 | 3650000 | 10.2 | 29.5 | 80.8 | 27.9 | 34.6 | 188000 | | | 8.56 |
| 27 | 7 | F | 23800 | 2900000 | 8.3 | 22.2 | 76.6 | 28.6 | 37.4 | 594000 | | | 9.18 |
| 28 | 20 | M | 3800 | 3010000 | 9.8 | 28.3 | 94.0 | 32.6 | 34.6 | 67000 | | | 8.74 |
| 29 | 20 | F | 9500 | 3640000 | 9.7 | 30.9 | 84.9 | 26.6 | 31.4 | 344000 | 46 | 15.6 | 5.05 |
| 30 | 64 | M | 800 | 2020000 | 6.8 | 18.7 | 92.6 | 33.7 | 36.4 | 17000 | | | 9.59 |
| 31 | 5 | F | 13600 | 2450000 | 6.6 | 21.1 | 86.1 | 26.9 | 31.3 | 340000 | | | 4.19 |

| Patient code | Age (year) | Sex | WBC per µl | RBC per µl | Hb gm/dl | HCT % | MCV fl | MCH pg | MCHC gm/dl | PLT | RDW_ SD (fl) | RDW_ CV (%) | Total prot-ein (g/dl) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 32 | 0.25 | M | 5500 | 3990000 | 10.6 | 31.7 | 79.4 | 26.6 | 33.4 | 453000 | | | 8.16 |
| 33 | 20 | F | 20600 | 3800000 | 11.9 | 36.2 | 95.0 | 34.0 | 35.5 | 12000 | | | 6.84 |
| 34 | 0.45 | F | 4200 | 3640000 | 9.9 | 27.3 | 75.0 | 27.2 | 36.3 | 344000 | | | 7.58 |
| 35 | 3.5 | M | 5300 | 3220000 | 8.9 | 25.4 | 78.9 | 27.6 | 35.0 | 547000 | | | 5.57 |
| 36 | 1.16 | M | 22500 | 4520000 | 7.8 | 26.1 | 57.7 | 17.3 | 29.9 | 897000 | | | 10.04 |
| 37 | 2.16 | M | 5100 | 2240000 | 5.9 | 18.2 | 81.3 | 26.3 | 32.4 | 67000 | | | 8.85 |
| 38 | 6 | F | 12000 | 2580000 | 7.1 | 23.5 | 91.1 | 27.5 | 30.2 | 799000 | | | 7.03 |
| 39 | 4.45 | F | 6900 | 3510000 | 10.5 | 28.6 | 81.5 | 29.9 | 36.7 | 328000 | | | 10.1 |
| 40 | 0.58 | F | 9300 | 3920000 | 10.7 | 28.3 | 72.2 | 27.3 | 37.8 | 391000 | | | 4.89 |
| 41 | 5 | F | 6700 | 2810000 | 8.3 | 24.2 | 86.1 | 29.5 | 34.3 | 218000 | | | 9.89 |
| 42 | 10 | M | 8700 | 4110000 | 9.7 | 31.1 | 75.4 | 23.6 | 31.3 | 72000 | | | 9.58 |
| 43 | 28 | F | 4800 | 4500000 | 11.8 | 36.0 | 80.0 | 26.2 | 32.5 | 197000 | | | 8.56 |
| 44 | 5 | M | 4700 | 3310000 | 8.6 | 24.6 | 74.3 | 26.0 | 35.0 | 106000 | | | 6.63 |
| 45 | 1.45 | M | 11500 | 3500000 | 9.6 | 27.9 | 79.7 | 27.4 | 34.4 | 574000 | | | 7.88 |
| 46 | 68 | F | 1220 | 2230000 | 7.5 | 23.2 | 104 | 33.6 | 32.3 | 72000 | | | 4.30 |
| 47 | 40 | F | 4700 | 1350000 | 5.5 | 14.1 | 104.4 | 40.7 | 39.0 | 147000 | 85.9 | 25.8 | 9.00 |

| Patient code | Age (year) | Sex | WBC per µl | RBC per µl | Hb gm/dl | HCT % | MCV fl | MCH pg | MCHC gm/dl | PLT | RDW_ SD (fl) | RDW_ CV (%) | Total protein (g/dl) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 48 | 52 | M | 3100 | 3980000 | 9.4 | 31.2 | 78.4 | 23.6 | 30.1 | 27000 | | | 8.42 |
| 49 | 6 | F | 1200 | 2670000 | 8.6 | 23.6 | 89.5 | 32.2 | 36.0 | 191000 | | | 6.01 |
| 50 | 1.5 | M | 3200 | 4120000 | 10.5 | 29.6 | 71.8 | 25.5 | 35.5 | 446000 | | | 4.47 |
| 51 | 1.58 | M | 6500 | 4210000 | 6.6 | 26.9 | 63.9 | 15.7 | 24.5 | 634000 | | | 6.67 |
| 52 | 10 | M | 9400 | 3660000 | 8.0 | 27.0 | 73.8 | 21.9 | 29.6 | 86000 | | | 12.6 |
| 53 | 12 | M | 250900 | 990000 | 5.0 | 10.1 | 102.0 | 50.5 | 49.5 | 331000 | | | 9.63 |
| 54 | 6 | F | 8400 | 3860000 | 10.0 | 31.1 | 80.6 | 25.9 | 32.2 | 137000 | | | 9.82 |
| 55 | 10 | M | 5500 | 4760000 | 12.8 | 41.1 | 88.0 | 27.7 | 32.1 | 131000 | 53.9 | 16.6 | 8.35 |
| 56 | 5 | M | 11000 | 3260000 | 4.8 | 20.2 | 62.0 | 14.7 | 23.8 | 626000 | | | 6.16 |
| 57 | 12 | M | 5500 | 3360000 | 12.0 | 34.5 | 102.7 | 35.7 | 34.8 | 256000 | | | 6.77 |
| 58 | 2.25 | M | 8900 | 3650000 | 7.7 | 25.8 | 70.7 | 21.1 | 29.8 | 369000 | | | 5.58 |
| 59 | 6 | M | 5700 | 3990000 | 10.8 | 31.5 | 78.9 | 27.1 | 34.3 | 185000 | | | 7.96 |
| 60 | 2 | M | 5200 | 2830000 | 4.1 | 18.8 | 66.4 | 15.5 | 21.8 | 275000 | | | 4.72 |
| 61 | 24 | F | 4100 | 3450000 | 12.0 | 34.1 | 98.8 | 34.8 | 35.2 | 58000 | 45.1 | 13.5 | 7.60 |
| 62 | 20 | M | 3300 | 4450000 | 12.8 | 41.0 | 104.7 | 35.7 | 34.1 | 68000 | | | 6.47 |
| 63 | 4 | M | 6900 | 3540000 | 7.0 | 24.2 | 68.4 | 19.8 | 28.9 | 533000 | | | 6.33 |

| Patient code | Age (year) | Sex | WBC per µl | RBC per µl | Hb gm/dl | HCT % | MCV fl | MCH pg | MCHC gm/dl | PLT | RDW_ SD (fL) | RDW_ CV (%) | Total prot-ein (g/dl) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 64 | 3 | M | 5400 | 4740000 | 8.9 | 31.0 | 65.4 | 18.8 | 28.7 | 229000 | | | 7.96 |
| 65 | 3.33 | F | 9200 | 1650000 | 4.2 | 13.9 | 84.2 | 25.5 | 30.2 | 141000 | 50.8 | 19.0 | 4.47 |
| 66 | 1.75 | F | 11100 | 2970000 | 4.2 | 17.3 | 58.2 | 14.1 | 24.3 | 329000 | | | 7.23 |
| 67 | 8 | M | 1800 | 1910000 | 3.4 | 12.6 | 66.0 | 17.8 | 27.0 | 71000 | | | 3.02 |
| 68 | 8 | F | 13200 | 910000 | 3.3 | 11.1 | 122.0 | 36.3 | 29.7 | 275000 | | | 8.64 |
| 69 | 1.5 | M | 6300 | 4780000 | 7.7 | 29.5 | 61.7 | 16.1 | 26.1 | 277000 | | | 7.23 |
| 70 | 1.25 | F | 10800 | 3890000 | 10.2 | 31.9 | 82.0 | 26.2 | 32.0 | 263000 | | | 4.94 |
| 71 | 1.75 | M | 10300 | 1900000 | 4.0 | 15.1 | 79.5 | 21.1 | 26.5 | 77000 | | | 7.76 |
| 72 | 7 | M | 8200 | 3090000 | 6.9 | 23.5 | 76.1 | 22.3 | 29.4 | 256000 | | | 4.90 |
| 73 | 64 | M | 7500 | 5330000 | 12.4 | 40.0 | 86.3 | 27.0 | 31.3 | 31000 | 47.8 | 15.1 | 6.69 |
| 74 | 50 | F | 9400 | 5250000 | 12.0 | 37.0 | 70.1 | 22.4 | 32.4 | 603000 | | | 7.19 |
| 75 | 21 | F | 8300 | 3320000 | 10.6 | 34.8 | 104.8 | 31.9 | 30.5 | 227000 | | | 4.88 |
| 76 | 68 | M | 8100 | 4320000 | 12.9 | 39.2 | 90.5 | 30.3 | 33.1 | 145000 | | | 7.97 |
| 77 | 36 | F | 6400 | 4130000 | 11.6 | 36.0 | 88.0 | 29.3 | 33.2 | 64000 | | | 6.51 |
| 78 | 1.83 | F | 8900 | 3400000 | 8.0 | 26.7 | 78.5 | 23.5 | 30.0 | 124000 | | | 4.96 |
| 79 | 3 | M | 3000 | 3380000 | 7.5 | 25.0 | 74.0 | 22.2 | 30.0 | 146000 | | | 6.99 |

| Patient code | Age (year) | Sex | WBC per µl | RBC per µl | Hb gm/dl | HCT % | MCV fl | MCH pg | MCHC gm/dl | PLT | RDW_ SD (fl) | RDW_ CV (%) | Total prot-ein (g/dl) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 80 | 11 | M | 2500 | 3820000 | 11.2 | 33.5 | 90.3 | 31.9 | 35.4 | 155000 | | | 6.35 |
| 81 | 40 | M | 4900 | 2730000 | 9.6 | 30.1 | 110.3 | 35.2 | 31.9 | 70000 | | | 5.74 |
| 82 | 20 | M | 2200 | 1400000 | 4.6 | 12.6 | 90.0 | 32.9 | 36.5 | 5000 | | | 6.69 |
| 83 | 7 | F | 3000 | 409000 | 11.0 | 32.6 | 79.7 | 26.9 | 33.7 | 20000 | | | 4.02 |
| 84 | 8 | F | 6400 | 2380000 | 7.3 | 21.2 | 89.1 | 30.7 | 34.4 | 212000 | | | 7.53 |
| 85 | 7 | M | 4800 | 3730000 | 10.8 | 31.1 | 83.4 | 29.0 | 34.7 | 228000 | | | 6.76 |
| 86 | 6 | M | 6200 | 3910000 | 8.6 | 28.1 | 71.9 | 22.0 | 30.6 | 460000 | | | 8.30 |
| 87 | 6 | M | 8400 | 3980000 | 10.5 | 31.1 | 78.1 | 26.4 | 33.8 | 183000 | | | 7.86 |
| 88 | 18 | M | 3800 | 3710000 | 12.8 | 38.0 | 102.4 | 34.5 | 33.7 | 108000 | | | 9.32 |
| 89 | 66 | M | 6500 | 4270000 | 12.9 | 40.0 | 93.2 | 31.3 | 32.6 | 215000 | | | 8.14 |
| 90 | 20 | M | 9400 | 2200000 | 7.8 | 22.9 | 104.1 | 35.5 | 34.1 | 172000 | | | 7.92 |
| 91 | 34 | M | 7100 | 4500000 | 12.7 | 39.1 | 87.0 | 28.2 | 32.4 | 222000 | 45.6 | 13.5 | 8.61 |
| 92 | 17 | M | 6200 | 4500000 | 12.8 | 39.2 | 87.2 | 28.6 | 32.6 | 142000 | | | 5.66 |
| 93 | 12 | F | 2300 | 1200000 | 3.0 | 10.1 | 84.2 | 25.0 | 29.7 | 87000 | | | 5.46 |
| 94 | 1.58 | F | 6600 | 2900000 | 6.3 | 22.0 | 75.9 | 21.7 | 28.6 | 102000 | | | 8.89 |
| 95 | 5 | F | 8300 | 4160000 | 11.7 | 34.2 | 82.2 | 28.1 | 34.2 | 192000 | 42.9 | 14.0 | 5.82 |
| 96 | 13 | M | 5800 | 4520000 | 13.0 | 38.5 | 87.4 | 29.9 | 34.2 | 48000 | 46.1 | 14.8 | 6.57 |

| Patient code | Age (Year) | Sex | WBC per µl | RBC per µl | Hb gm/dl | HCT % | MCV fl | MCH pg | MCHC gm/dl | PLT | RDW_ SD (fl) | RDW_ CV (%) | Total prot-ein (g/dl) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 97 | 1.92 | M | 4400 | 4130000 | 10.1 | 32.4 | 78.5 | 24.5 | 31.2 | 345000 | 51.5 | 17.7 | 6.87 |
| 98 | 9 | F | 4700 | 3550000 | 10.7 | 28.0 | 78.9 | 30.1 | 38.2 | 282000 | | | 7.46 |
| 99 | 0.83 | M | 10600 | 5120000 | 8.8 | 28.7 | 56.1 | 17.2 | 30.7 | 384000 | | | 7.46 |
| 100 | 0.5 | F | 6200 | 3780000 | 9.2 | 25.8 | 68.3 | 24.3 | 35.7 | 275000 | | | 5.08 |
| 101 | 12 | M | 9300 | 4290000 | 8.9 | 30.2 | 70.4 | 20.7 | 29.5 | 517000 | 42.1 | 16.0 | 7.98 |
| 102 | 27 | F | 8100 | 4150000 | 12.0 | 38.0 | 91.5 | 28.9 | 31.5 | 182000 | 42.2 | 13.4 | 6.73 |
| 103 | 2.66 | F | 12300 | 4070000 | 11.6 | 35.0 | 86.0 | 28.5 | 33.1 | 267000 | 48.1 | 15.5 | 7.44 |
| 104 | 53 | F | 6400 | 3440000 | 11.4 | 33.0 | 95.0 | 33.1 | 34.5 | 53000 | 56.4 | 17.3 | 9.93 |
| 105 | 45 | F | 6200 | 4040000 | 12.0 | 37.2 | 92.1 | 30.0 | 32.4 | 165000 | 48.7 | 15.3 | 9.21 |
| 106 | 45 | F | 6500 | 4110000 | 11.9 | 36.8 | 89.5 | 29.3 | 32.6 | 188000 | 42.4 | 12.5 | 7.13 |
| 107 | 38 | F | 3500 | 4010000 | 12.0 | 37.0 | 92.6 | 30.0 | 32.8 | 102000 | 45.6 | 13.1 | 10.13 |
| 108 | 50 | M | 6600 | 4120000 | 12.9 | 39.0 | 94.0 | 31.6 | 33.3 | 137000 | 45.4 | 13.5 | 7.38 |
| 109 | 25 | F | 6300 | 3160000 | 10.2 | 28.1 | 88.9 | 32.0 | 36.3 | 298000 | | | 5.92 |
| 110 | 32 | F | 2800 | 3560000 | 10.6 | 31.2 | 87.6 | 29.8 | 34.0 | 20000 | 50.4 | 17.3 | 8.40 |
| 111 | 4 | M | 5900 | 3610000 | 9.9 | 30.6 | 84.8 | 27.4 | 32.4 | 153000 | 44.2 | 14.2 | 8.22 |
| 112 | 23 | F | 4100 | 5030000 | 9.0 | 32.9 | 65.4 | 17.9 | 27.4 | 120000 | 42.2 | 17.1 | 8.81 |
| 113 | 29 | F | 3000 | 4000000 | 7.8 | 28.1 | 70.3 | 19.5 | 27.8 | 204000 | 49.2 | 19.7 | 8.22 |

Notes:- Empty rows for RDW are values that are not determined.

## Annex V Morphology of erythrocytes, Solubility test and electrophoresis.

| Patients Code | Solubility test | Malarial Parasite | Morphology of RBC | Electrophoresis (PAGE) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | HbA | HbF | $HbA_2$ | HbS | UB |
| 1 | -ve | -ve | Variable size, hypochromic | 1 | 1 | 1 | 0 | 1 |
| 2 | -ve | -ve | Microcytic, small size | 1 | 1 | 1 | 0 | 1 |
| 3 | -ve | -ve | Anisocytosis (variable size) | 1 | 1 | 1 | 0 | 1 |
| 4 | -ve | -ve | Normochromic, Anisocytosis | 1 | 1 | 1 | 0 | 1 |
| 5 | -ve | -ve | Hypochromic, anisocytosis | 1 | 1 | 1 | 0 | 1 |
| 6 | -ve | -ve | Anisocytosis, hypochromic | 1 | 1 | 1 | 0 | 1 |
| 7 | -ve | -ve | Anisocytosis, hyochromic | 1 | 1 | 1 | 0 | 1 |
| 8 | -ve | -ve | Variable size (ancanthocyte, tear drop, etc.) | 1 | 1 | 1 | 0 | 1 |
| 9 | -ve | -ve | Anisocytosis, hypochromic | 1 | 1 | 1 | 0 | 1 |
| 10 | -ve | -ve | Anisocytosis (tear drop, helment, ancanthocyte, etc.), poikilocytosis | 1 | 1 | 1 | 0 | 1 |
| 11 | -ve | -ve | Normocytic | 1 | 1 | 1 | 0 | 1 |
| 12 | -ve | -ve | Hypochromic, anisocytosis, most are large size RBC (spherocytosis) | 1 | 1 | 1 | 0 | 1 |
| 13 | -ve | -ve | Many ancanthocytes, few tear drops generally anisocytosis | 1 | 1 | 1 | 0 | 1 |
| 14 | -ve | -ve | Hypochromic, variable size large and small. | 1 | 1 | 1 | 0 | 1 |
| 15 | -ve | -ve | Normocytic but there are few microcytic RBC | 1 | 1 | 1 | 0 | 1 |
| 16 | -ve | -ve | Normocytic | 1 | 1 | 1 | 0 | 1 |

| Patients Code | Solubility test | Malarial Parasite | Morphology of RBC | Electrophoresis (PAGE) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | HbA | HbF | $HbA_2$ | HbS | UB |
| 17 | -ve | -ve | Hypochromic, normocytic | 1 | 1 | 1 | 0 | 1 |
| 18 | -ve | -ve | Normocytic, normochromic | 1 | 1 | 1 | 0 | 1 |
| 19 | -ve | -ve | Anisocytosis (variable size), hypochromic. | 1 | 1 | 1 | 0 | 1 |
| 20 | -ve | -ve | Variable size (many ancanthocyte, hypochromic | 1 | 1 | 1 | 0 | 1 |
| 21 | -ve | -ve | Anisocytosis (tear drop, oval shape, helmet cell, etc), hypochromic. | 1 | 1 | 1 | 0 | 1 |
| 22 | -ve | -ve | Variable size, normochromic, there are cells that are also hypochromic | 1 | 1 | 1 | 0 | 1 |
| 23 | -ve | -ve | Normocytic, hypochromic | 1 | 1 | 1 | 0 | 1 |
| 24 | -ve | -ve | Hypochromic, Variable size | 1 | 1 | 1 | 0 | 1 |
| 25 | -ve | -ve | hypochromic, cells that seems sickle, most are small size | 1 | 1 | 1 | 0 | 1 |
| 26 | -ve | -ve | Hypochromic, Variable size | 1 | 1 | 1 | 0 | 1 |
| 27 | -ve | -ve | Hypochromic, normal shape but variable size | 1 | 1 | 1 | 0 | 1 |
| 28 | -ve | -ve | Normochromic, Variable size (tear drop) | 1 | 1 | 1 | 0 | 1 |
| 29 | -ve | -ve | Normochromic Variable size (many Anchanthocytes) | 1 | 1 | 1 | 0 | 1 |
| 30 | -ve | -ve | Hypochromic, (the RBC is completely white, many small RBC, few large). | 1 | 1 | 1 | 0 | 1 |
| 31 | -ve | -ve | Hypochromic, Variable size | 1 | 1 | 1 | 0 | 1 |
| 32 | -ve | -ve | Hypochromic, Variable size | 1 | 1 | 1 | 0 | 1 |
| 33 | -ve | -ve | Normocytic but there are some ancanthocytes, hypochromic | 1 | 1 | 1 | 0 | 1 |
| 34 | -ve | -ve | Many acanthocytes, few tear drops (variable size) | 1 | 1 | 1 | 0 | 1 |

| Patients Code | Solubility test | Malarial Parasite | Morphology of RBC | Electrophoresis (PAGE) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | HbA | HbF | $HbA_2$ | HbS | UB |
| 35 | -ve | -ve | Variable size, helments, etc, Hypochromic | 1 | 1 | 1 | 0 | 1 |
| * 36 | +ve | -ve | Variable size and shape | 1 | 1 | 1 | 0 | 1 |
| • 37 | -ve | +ve | Hypochromic, microcytic. | 1 | 1 | 1 | 0 | 1 |
| * 38 | +ve | -ve | Hypochromic, variable size and shape (anisopoikilocytosis). | 1 | 1 | 1 | 0 | 1 |
| 39 | -ve | -ve | Hypochromic normocytic (normal size) | 1 | 1 | 1 | 0 | 1 |
| 40 | -ve | -ve | Hypochromic normal shape, variable size (large and small) | 1 | 1 | 1 | 0 | 1 |
| 41 | -ve | -ve | Hypochromic, variable size | 1 | 1 | 1 | 0 | 1 |
| 42 | -ve | -ve | Hypochromic, variable size | 1 | 1 | 1 | 0 | 1 |
| 43 | -ve | -ve | Normochromic Variable size (spherocytes) | 1 | 1 | 1 | 0 | 1 |
| 44 | -ve | -ve | Hypochromic, variable size | 1 | 1 | 1 | 0 | 1 |
| * 45 | +ve | -ve | Elliptocytosis, spherocytes, small and large RBC, hypochromic. | 1 | 1 | 1 | 0 | 1 |
| 46 | -ve | -ve | Hypochromic, variable size and shape | 1 | 1 | 1 | 0 | 1 |
| 47 | -ve | -ve | Hypochromic, normocytic | 1 | 1 | 1 | 0 | 1 |
| 48 | -ve | -ve | Normchromic variable shape | 1 | 1 | 1 | 0 | 1 |
| 49 | -ve | -ve | Hypochromic | 1 | 1 | 1 | 0 | 1 |
| 50 | -ve | -ve | Hypochromic, variable size and shape (few ancanthocytes) | 1 | 1 | 1 | 0 | 1 |
| 51 | -ve | -ve | Hypochromic, variable size and shape. | 1 | 1 | 1 | 0 | 1 |
| 52 | -ve | -ve | Hypochromic, variable size. | 1 | 1 | 1 | 0 | 1 |

| Patients Code | Solubility test | Malarial Parasite | Morphology of RBC | Electrophoresis (PAGE) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | HbA | HbF | $HbA_2$ | HbS | UB |
| 53 | -ve | -ve | full of lymphocytes, few RBC that are small, large and hypochromic | 1 | 1 | 1 | 0 | 1 |
| 54 | -ve | -ve | Hypochromic, variable size and shape. | 1 | 1 | 1 | 0 | 1 |
| ♦55 | -ve | -ve | Hypochromic, variable size (spherocytes, stomatocyte, irregular shape RBC) | 1 | 1 | 1 | 0 | 1 |
| 56 | -ve | -ve | Hypochromic, variable size and shape. | 1 | 1 | 1 | 0 | 1 |
| 57 | -ve | -ve | Normochromic variable size and shape | 1 | 1 | 1 | 0 | 1 |
| 58 | -ve | -ve | Hypochromic, variable size and shape. | 1 | 1 | 1 | 0 | 1 |
| 59 | -ve | -ve | Hypochromic, variable size. | 1 | 1 | 1 | 0 | 1 |
| 60 | -ve | -ve | Hypochromic, variable size and shape. | 1 | 1 | 1 | 0 | 1 |
| 61 | -ve | -ve | Normchromic variable shape | 1 | 1 | 1 | 0 | 1 |
| 62 | -ve | -ve | Normochromic, variable size. | 1 | 1 | 1 | 0 | 1 |
| 63 | -ve | -ve | Hypochromic, variable size and shape. | 1 | 1 | 1 | 0 | 1 |
| 64 | -ve | -ve | Hypochromic, variable size and shape. | 1 | 1 | 1 | 0 | 1 |
| 65 | -ve | -ve | Hypochromic, variable size and shape. | 1 | 1 | 1 | 0 | 1 |
| 66 | -ve | -ve | Microcytic, hypochromic | 1 | 1 | 1 | 0 | 1 |
| 67 | -ve | -ve | Hypochromic, variable size and shape | 1 | 1 | 1 | 0 | 1 |
| 68 | -ve | -ve | Hypochromic, variable size, there are spherocytes. | 1 | 1 | 1 | 0 | 1 |
| 69 | -ve | -ve | Hypochromic, variable size and shape. | 1 | 1 | 1 | 0 | 1 |
| 70 | -ve | -ve | Hypochromic, variable size and shape (many spur cell). | 1 | 1 | 1 | 0 | 1 |

| Patients Code | Solubility test | Malarial Parasite | Morphology of RBC | Electrophoresis (PAGE) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | HbA | HbF | $HbA_2$ | HbS | UB |
| * 71 | +ve | -ve | Hypochromic, variable size. | 1 | 1 | 1 | 0 | 1 |
| 72 | -ve | -ve | Hypochromic, variable size and shape. | 1 | 1 | 1 | 0 | 1 |
| 73 | -ve | -ve | Normochromic, normocytic. | 1 | 1 | 1 | 0 | 1 |
| 74 | -ve | -ve | Normochromic, anisocytosis and poikilocytosis (many Spur cell) | 1 | 1 | 1 | 0 | 1 |
| 75 | -ve | -ve | Normochromic, anisopoikilocytosis (many Spure cell, tear drop) | 1 | 1 | 1 | 0 | 1 |
| 76 | -ve | -ve | Normochromic, There are spure cell, The RBC are large | 1 | 1 | 1 | 0 | 1 |
| 77 | -ve | -ve | Normochromic, most RBC's are large | 1 | 1 | 1 | 0 | 1 |
| 78 | -ve | -ve | Hypochromic, variable size. | 1 | 1 | 1 | 0 | 1 |
| 79 | -ve | -ve | Hypochromic, variable size. | 1 | 1 | 1 | 0 | 1 |
| 80 | -ve | -ve | Normochromic, variable shape | 1 | 1 | 1 | 0 | 1 |
| 81 | -ve | -ve | Hypochromic, variable size and shape. | 1 | 1 | 1 | 0 | 1 |
| 82 | -ve | -ve | Hypochromic, variable size. | 1 | 1 | 1 | 0 | 1 |
| 83 | -ve | -ve | Normochromic, variable shape and size. | 1 | 1 | 1 | 0 | 1 |
| 84 | -ve | -ve | Hypochromic, variable size. | 1 | 1 | 1 | 0 | 1 |
| 85 | -ve | -ve | Hypochromic, variable size. | 1 | 1 | 1 | 0 | 1 |
| 86 | -ve | -ve | Hypochromic, variable size and shape. | 1 | 1 | 1 | 0 | 1 |
| 87 | -ve | -ve | Hypochromic, variable size. | 1 | 1 | 1 | 0 | 1 |
| 88 | -ve | -ve | Normochromic, Large size RBC | 1 | 1 | 1 | 0 | 1 |

| Patients Code | Solubility test | Malarial Parasite | Morphology of RBC | Electrophoresis (PAGE) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | HbA | HbF | $HbA_2$ | HbS | UB |
| 89 | -ve | -ve | Normochromic, variable size and shape (there are spherocyte) | 1 | 1 | 1 | 0 | 1 |
| 90 | -ve | -ve | Hypochromic, variable size and shape. | 1 | 1 | 1 | 0 | 1 |
| 91 | -ve | -ve | Normochromic, Spherocytes | 1 | 1 | 1 | 0 | 1 |
| ♦92 | -ve | -ve | Normochromic and some are hypochromic, variable size. | 1 | 1 | 1 | 1 | 1 |
| 93 | -ve | -ve | Hypochromic, variable size and shape. | 1 | 1 | 1 | 0 | 1 |
| * 94 | +ve | -ve | Hypochromic, variable size. | 1 | 1 | 1 | 0 | 1 |
| 95 | -ve | -ve | Variable size, some spherocytes, some hypochromic cell. | 1 | 1 | 1 | 0 | 1 |
| 96 | -ve | -ve | Normochromic, variable size and shape (spherocyte, tear drop, etc). | 1 | 1 | 1 | 0 | 1 |
| ♦97 | -ve | -ve | Hypochromic, variable size and shape. (Spur cell). | 1 | 1 | 1 | 1 | 1 |
| 98 | -ve | -ve | Normochromic, variable size | 1 | 1 | 1 | 0 | 1 |
| 99 | -ve | -ve | Hypochromic, variable size and shape (many spur cell). | 1 | 1 | 1 | 0 | 1 |
| 100 | -ve | -ve | Hypochromic, variable size and shape | 1 | 1 | 1 | 0 | 1 |
| 101 | -ve | -ve | Hypochromic, Variable shape (Anacanthocytes, spherocytes, etc). | 1 | 1 | 1 | 0 | 1 |
| 102 | -ve | -ve | Hypochromic, variable size and shape (spherocytes) | 1 | 1 | 1 | 0 | 1 |
| 103 | -ve | -ve | Normochromic, variable size and shape. (Some are Hypochromic) | 1 | 1 | 1 | 0 | 1 |
| 104 | -ve | -ve | Normochromic, macrocytic (spherocytes), Normal shape | 1 | 1 | 1 | 0 | 1 |
| 105 | -ve | -ve | Hypochromic, most are normal shape (there are spherocytes) | 1 | 1 | 1 | 0 | 1 |
| 106 | -ve | -ve | Hypochromic, Variable shape (ancanthocytes), microcytic | 1 | 1 | 1 | 0 | 1 |

| Patients Code | Solubility test | Malarial Parasite | Morphology of RBC | Electrophoresis (PAGE) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | HbA | HbF | $HbA_2$ | HbS | UB |
| 107 | -ve | -ve | Normochromic, Microcytic | 1 | 1 | 1 | 0 | 1 |
| 108 | -ve | -ve | Normochromic, Variable size (there are anacanthocytes) | 1 | 1 | 1 | 0 | 1 |
| 109 | -ve | -ve | Hypochromic, Variable size | 1 | 1 | 1 | 0 | 1 |
| 110 | -ve | -ve | Normochromic, Macrocytic. | 1 | 1 | 1 | 0 | 1 |
| 111 | -ve | -ve | Variable size and Shape (full of acanthocytes) | 1 | 1 | 1 | 0 | 1 |
| 112 | -ve | -ve | Hypochromic, variable size and Shape | 1 | 1 | 1 | 0 | 1 |
| 113 | -ve | -ve | Hypochromic, Variable size and Shape | 1 | 1 | 1 | 0 | 1 |

Remarks 1 – presence of band
0 – absence of band
UB – unknown band

**Legend:-**

*Positive solubility test
♦Have HbS on electrophoresis
• Malaria positive

# Annex VI P r o t o c o l s

## 1. Sample collection

1-2 ml of blood was collected from each donor. One drop was used to prepare blood film. The rest were used for Hb isolation

## 2. Morphological analysis

Chemicals and reagents needed

- Wright stain
- Slide and slide box
- Microscope
- Immersion oil

A drop of whole blood was applied on the slide. Using a second clean slide, a spreader, touch the drop of blood with it and spread the blood to the edge. Dry the slide and label it.

Put it in a leveled position and pour the Wright stain on it and cover it totally. Wait fro 2-3 minutes. Wash it with distilled water and dry it. Look under microscope with oil immersion objective (100x) for morphology abnormalities or parasites.

## 3. Preparation of Hemolysate from whole blood

Whole blood taken by vein puncture was centrifuged in refrigerated centrifuge at a speed of 3500 RPM for 5 minutes in order to separate the RBC from the serum. Serum was removed and RBC was washed three times with saline (0.9%) After each wash the

sample were centrifuged at 3500 RPM for 10 minutes. To the washed RBC add one volume of distilled water and add carbon tetrachloride at ¼ solution volume. Mix at high speed with vortex mixer for one minute. After that centrifuge the solution at a speed of 3500 RPM for 10 minutes. Remove the upper red solution (the hemolysate) and pour in an eppindorf tube and store it at -70$^{o}$C until used.

## 4. Solubility test

1. Saponin
2. Potassium phosphate ($K_2HPO_4$/ $KH_2PO_4$)
3. Sodium dithionite ($Na_2S_2O4$)

Weigh

- 216 gm anhydrous Dibasic Potassium phosphate
- 169 gm monobasic Potassium phosphate
- 10gm Sodium dithionite $Na_2S_2O_4$
- 1gm Saponin

Dissolve in distilled water and raise the volume up to1 liter and store the regent in a refrigerator.

- 20 µl of anticoagulated whole blood is mixed with 2 ml reagent in a glass test tube.
- Mix and let it stand for 5 minutes

- Hold the tube 2.5 cm in front of a white card with a black line and look for transparency.

**Interpretaion**

- If the black line is visible the test is negative that is there is no sickle Hb in the patients blood.
- If the black line is not visible the test is positive

# 5. Protocol for Native-PAGE

## 5.1 Chemicals and reagents used

1. Acryl amide /Bisacryl amide 40%
2. Tris [Hydroxy methyl]- amino methane (Tris Base)
3. Glycerol
4. Ammonium persulphate 10%
5. Tetramethyl ethylene diamine (TEMED)
6. Glycine
7. Hemoglobin Control /AFSC.
8. Ethylenediaminetetreacetic acid (EDTA)
9. Sodium Chloride
10. Hydrochloric acid
11. Bromophenol blue

## 5.2 Preparation of buffers for Native PAGE

### 5.2.1 Preparation of running gel buffer pH 8.8 (2.25M Tris base)

27.23gm Tris base was dissolve in distilled water and the pH adjusted to 8.8 with 1M or 6M HCl and prepares 100ml of solution

### 5.2.2 Preparation of stacking gel buffer pH 6.8 (0.75M Tris base)

4.5 gm Tris base was dissolved in distilled water and the pH adjusted to 6.8 with 1 M or 6M HCl to prepare 50 ml of solution.

### 5.2.3 Preparation of electrophoresis buffer pH 8.3 (0.75M Tris base)

- 15gm of Tris base
- 72gm of Glycine

were dissolved in 5 liter of distilled water, pH was adjusted to 8.3 with 6M HCl

## 5.3 Preparation of acryl amide resolving gel

Mix together

- Acryl amide/bis acryl amide (19:1) solution----------7.5ml
- Running gel buffer pH 8.8----------3.33ml
- Glycerol ----------4.5gm
- TEMED ----------20µl
- Distilled water ----------5.33ml
- Ammonium persulphate (10%) solution ----------167µl

Mix well and pour it to the gel cast with a pasture pipette immediately other wise it will congeal.

## 5.4 Preparation of stacking gel from acryl amide

Mix together

- Acryl amide/bis acryl amide solution (19:1)----------1.25ml
- Stacking gel buffer pH 6.8----------1.67ml
- TEMED ---------- 10µl
- Distilled water ----------7.33ml
- Ammonium persulphate solution (10%) ---------- 100µl

Mix well and pour it to the gel cast on the top of the resolving gel with a pasture pipette. Immediately insert the comb and wait until it polymerize within 30 to 60 min.

## 5.5 Preparation of sample buffer for Native – PAGE sample

Mix:-

- 30 ml glycerol
- 18.8 ml stacking gel buffer
- 1.5 ml Bromophenol blue
- Make it to 100ml with Distilled water

Hemolysate is diluted with sample buffer containing bromophenol blue, glycerol, stacking gel buffer, and distilled water. The bromophenol blue is used to indicate the front and the glycerol to make the sample dense therefore sink into the bottom of the hole.

## 5.6 Preparation of staining and destaining chemicals

1 Methanol

2 Acetic acid

3 Coomassie brilliant Blue R 250

### 5.6.1 Staining Solution (0.025%)

Weigh 0.025 grams of Coomassie brilliant blue R 250 in a beaker

Add 40 ml methanol to it and wait for five minutes

Slowly add 7milliliter acetic acid and distilled water and make to volume with 100 ml volumetric flask. Mix it.

### 5.6.2 Destaining solution (5% methanol and 7% acetic acid)

Measure 50 ml methanol and 70 ml acetic acid and transfer to a 1-liter volumetric flask and make to a liter with distilled water and mix

## 5.7 Running the electrophoresis

The well is loaded with 5µl sample (hemolysate: sample buffer, 5µl: 100µl) and run for 20 hrs at 100 volts and 20 mA.

- When the running time is over dismantle the electrophoresis unit and remove the gel.
- Put the gel in staining dish and pour Coomassie brilliant blue over it.
- Put it on a horizontal shaker for two hours
- Decant Coomassie and pour destainning chemicals and put it again on the shaker

- Wash it repeatedly by changing the destaining chemicals several times
- Finally decant it and view the bands.

## 6. Agarose gel electrophoresis

### 6.1 Electrophoreis buffer preparation pH 8.6

Weigh

- Tris base 12 gm
- EDTA 1.22gm
- Boric acid 1.5gm

Dissolve in 1 liter distilled water and adjust the pH to 8.6 with boric acid.

### 6.2 Preparation of Agarose gel

- Weigh 2 gm agarose, add 200ml electrophoresis buffer and heat it until it dissolves.
- Allow the agarose to cool
- Prepare the horizontal gel cast and position the desired combs to cast slots.
- Pour the cooled liquid agarose into the gel cast and allow it to solidify.
- Place it in the electrophoresis chamber and carefully remove the comb.
- Load the 5µl hemolysate that is dilute with the electrophoresis buffer (5µl hemolysate: 100µl sample buffer)
- Add sufficient amount of the electrophoresis buffer to cover the gel
- Run the electrophoresis at 100 volts and for 6 hours.

- Remove the gel and stain it with Coomassie brilliant blue for 1 hour and destain it consecutively with 7% acetic acid and 5% methanol and View the bands.

## 7. Photography

**-** Blood film

- Gels

# Declaration

**I the undersigned, declare that this thesis is my original work, has not been presented for a degree in other University and that all sources of material used for the thesis have been duly acknowledged.**

**Name of Student _____ Signature_____**

**Date of submission _____**