"COMPARATIVE STUDY BETWEEN HPLC AND Hb ELECTROPHORESIS TO CHARACTERIZE HEMOGLOBIN PROFILE IN HEMOGLOBINOPATHIES"

By

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Dissertation submitted to

BLDE (DEEMED TO BE UNIVERSITY), Vijayapura, Karnataka

In partial fulfilment of the requirements for the award of the degree of

DOCTOR OF MEDICINE

IN

PATHOLOGY

Under the Guidance of

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ACKNOWLEDGEMENT

First and foremost, I thank God almighty, for blessing me with patience, consistence and immense strength which led me towards the completion of my work.

I would never have been able to finish my dissertation without the guidance of my respected teachers, support from my family and friends.

I would like to dedicate my dissertation to my grandfather **(Late Dr A Sanandachari)** who had always been my guiding light, my inspiration and my strength. He had always encouraged me to bring out the best in me. I thank my mother **(K Sakunthala)**, my father **(Dr K V S Rao)** and my brother **(K Kalyan)** for always being there for me whenever I needed them. Without their support and love I would not have been able to finish my work.

I take this opportunity to convey my gratitude to my guide **Dr R M Potekar** (MD Pathology), Professor Department of Pathology, Shri B.M.Patil Medical College, Hospital & Research Centre Vijayapura, Karnataka for his guidance and constant encouragement, throughout the journey of my research work. From patient selection to diagnosis he had always helped me. His knowledge of the subject and dedication towards his work are the two things which I always wanted to follow. Learning from him was a blissful experience.

I extend my sincere thanks to **Dr.Surekha U Arakeri** (Head of the Department, Department of Pathology, Shri B.M.Patil Medical College, Hospital & Research Centre, Vijayapura, Karnataka), Professors and Assistant Professors of Department of Pathology, **Dr. B.R. Yelikar** Prof, **Dr. S.B. Hippargi** Prof, **Dr. Mahesh H. Karigoudar** Prof, **Dr. Girija Patil** Assoc Prof, **Dr.Prakash M.Patil** Assoc Prof, **Dr. Vijayalaxmi S Patil** Assoc Prof**, Dr. Savitri M. Nerune** Assoc Prof, **Dr. Mamatha**

K. Assoc Prof**, Dr. Anil Reddy Konduru** Asst Prof, **Dr. Satish Arakeri** Asst Prof and **Dr**. **Sneha Jawalkar** Asst Prof; for their constant support and encouragement throughout the work.

I am grateful to my Co-guide **DR. M.M Patil**, (MD Paediatrics), Associate Professor Department Of Paediatrics , Shri B.M.Patil Medical College, Hospital &

Research Centre Vijayapura, Karnataka. It is my privilege to express my thanks for his selfless attitude, for giving me the freedom to experiment with my ideas and always being there for me, whenever I needed his help.

I would like to thank **Dr Vinita Naik** and **Dr Aeshita Singh**, my two mentors who were always there for me during my tough time. I would also like to thank **Dr Prachi Sinkar** for guiding me in selecting cases and interpreting electrophoresis graphs.

Special thanks to my freinds **Dr Shubham Chourishi, Dr Puneet Singh, Dr Neha Mahesh Bhosale, Dr Sahithya Hindupur** and **Dr Sohan Rao HR**, my seniors **Dr Nayantrishna Nath, Dr Sadiya Mirji and Dr Prashant Rajput** and my juniors.

Lastly I would like to thank **Mr.Praveen Kumar Honwad** and **Mr Mukades Rathod** for helping me in technical work.

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ABSTRACT

INTRODUCTION:

Mutation or deletion of one of the globin genes of hemoglobin results in thalassemias and hemoglobinopathies. Two diagnostic modalities which can be used to study hemoglobin abnormalities is Hemoglobin electrophoresis and capillary zone electrophoresis.

METHODS:

A total of 41 adults and children blood samples were examined for routine thalassemia screening using both the modalities, excluding those patients who had undergone blood transfusion within a span of 12weeks. All samples were analysed using automated cell counter (Sysmex XN-1000), HPLC (Biorad D-10) and capillary zone electrophoresis.

RESULTS:

Out of 41 cases, 24 cases were of thalassemia trait, 9 cases were normal, 3 cases of β-thalassemia major, 2 cases of homozygous/ double heterozygous for β-thalassemia and δβ-thalassemia, 1 case of sickle cell anemia, 1 case of Compound heterozygous sickle cell anemia and β-thalassemia and 1 case of compound heterozygous for HbE/ β-thalassemia.

When HbF value is >16.5% it merges with HbA1c values in HPLC(Bio-Rad D-10) because of which it is very difficult to comment about exact values of HbF with the help of HPLC. So for such cases CZE helped us to evaluate exact value of HbF.

CONCLUSIONS:

Forty one cases were collected in which both HPLC and Hb electrophoresis gave same results for 34 cases and remaining 7 cases we got different impressions from both the modalities.

Advantage of Hb electrophoresis over HPLC is that it gives exact values of HbF which is very essential to differentiate thalassemia intermedia from thalassemia major and also it exactly evaluates percentage of HbE which elutes with HbA in case of HPLC. CZE is complimentary to HPLC, which is very helpful for evaluating exact values of HbF,HbS and HbE.

LIST OF ABBREVATIONS USED

ARMS	-	Amplification Refractory Mutation System
β	-	Beta
CVS	-	Chorionic Villous Sampling
CZE	-	Capillary Zone Electrophoresis
EPO	-	Erythropoietin
G6PD	-	Glucose 6 Phosphate Dehydrogenase
Hb	-	Hemoglobin
HbA	-	Adult hemoglobin
HPFH	-	Hereditary Persistence of Fetal Hemoglobin
HPLC	-	High Performance Liquid Chromatography
IDA	-	Iron Decifiency Anemia
MCH	-	Mean corpuscular haemoglobin
Min.	-	Minute
No.	-	Number
PNH	-	Paroxysmal Nocturnal Haemoglobinuria
RNA	-	Ribonucleic acid
WHO	-	World Health Organisation
Wks	-	Weeks

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INTRODUCTION

Thalassemias and other hemoglobinopathies are a group of genetic disorders resulting from point mutation or deletion of one of the globin genes of hemoglobin[1]. It is the World's most common genetically inherited disorder[2]. Accurate quantification of different hemoglobin fractions are required to diagnosis these diseases[3]. Till date 1,200 Hb variants are detected[4].

Prevalence of thalassemias and other hemoglobinopathies in World:

Thalassemia is an autosomal recessive disease[5]. According to WHO around 5% of World's population are carriers for genetic hemoglobin disorder[2]. Approximately 1,00,000 thalassemia major cases are born every year worldwide[5].

Prevalence of thalassemias and other hemoglobinopathies in India:

In India frequency of β-thalassemia in general population is 3.5% to 15%[6]. On an average India is having >25million thalassemia carriers in which β-thalassemia, HbE/β-thalassemia and HbD-Punjab forms the major bulk[7]. 10% of total World thalassemia babies are born in India every year which is equivalent to around 10,000 new cases[3]. One of the reason for such a high count in India is consanguineous marriages[2].

Prevalence of β-thalassemia trait and sickle cell anemia in India is around 3-17% and 1-44% respectively[2].Whereas prevalence of β-thalassemia carriers in Karnataka is 2.16% [8].

According to National Family Health Survey (NFHS III) IDA is being the most common cause of anemia in pediatric age (prevalence being around 70%) followed by thalassemia. Because of which it becomes very essential to diagnose these two entities properly so that the child gets appropriate treatment[9].

Why this study is required?:

10,000 new cases of thalassemia major every year in India is a huge economic burden for the country as the management is expensive[2]. It costs around 90,000-1,00,000 for management of a child at around 3yrs of age, which keeps on increasing with increase in age[10].

Screening plays a very important role here as if this entity is diagnosed in early pregnancy then couple can be counseled to go for termination of pregnancy, but if it is diagnosed in late pregnancy or in neonatal life then parent's of the newborn can be educated regarding outcome of consecutive pregnancy.

OBJECTIVE OF THE STUDY

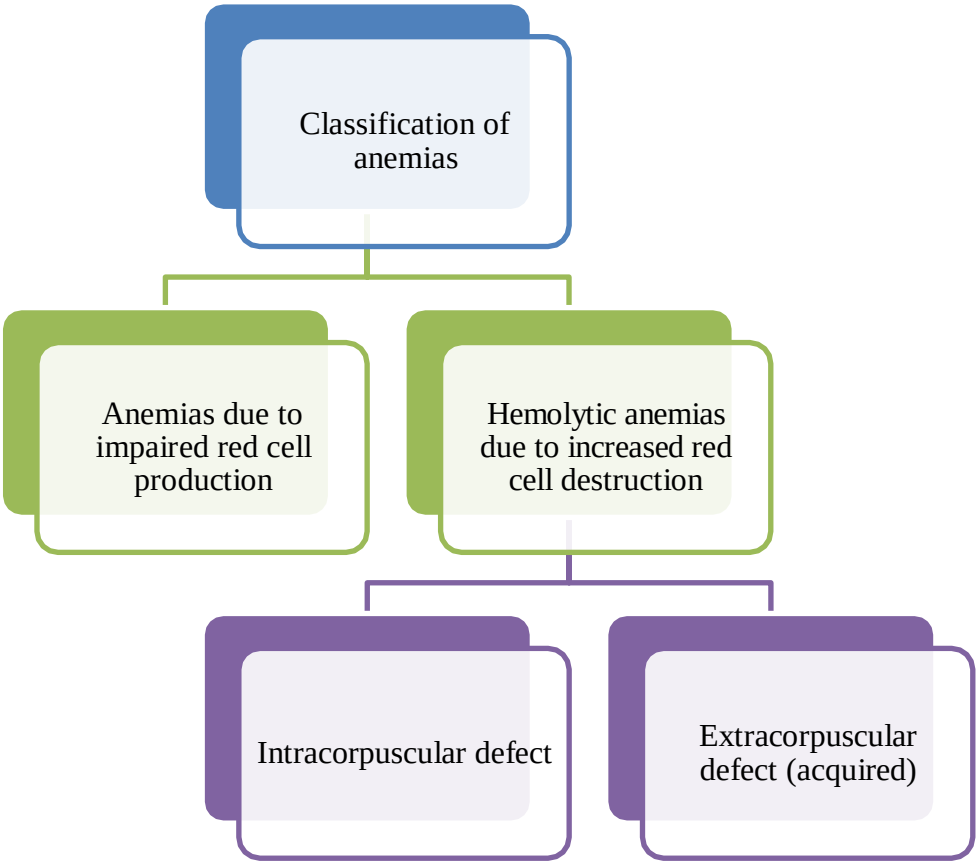
To compare 2 different modalities i.e. cation exchange HPLC and Hb electrophoresis (CapillaryZone Electrophoresis) to assess the characterization of hemoglobin profile in hemoglobinopathies.

REVIEW OF LITERATURE

Anemia:

Anemia is defined as a reduction of the total circulating red cell mass below normal limits[11].

Classification of anemia:



A. Anemias due to impaired red cell production:

- Deficiency of essential nutrients

  -Iron deficiency

  -Vitamin B12, Folate deficiency

  -Vitamin C deficiency

- Defect in stem cell/ erythroid precursor

  -Aplastic anemia

  -Pure red cell aplasia

- Miscellaneous

  -Anemia of chronic disorders

  -Marrow suppression due to drugs

B. Hemolytic anemias due to increased red cell destruction:

- Intracorpuscular defect

	Hereditary	Acquired
Enzyme deficiency	-G6PD deficiency	-PNH -Secondary to liver disease
Membrane defect	-Herediatry spherocytosis -Hereditary ovalocytosis	-Infections
Haemoglobin abnormalities	-Haemoglobinopathies a.Thalassemia syndromes b.Sickle syndromes c.HbD,E,etc	

- Extracorpuscular defect (acquired)

  -Immune haemolytic anemia

  -Fragmentation syndromes

  -Hypersplenism

Structure of RBC:

It is a biconcave, flexible disk: flattened and depressed in the centre with a dumbell-shaped cross section, made up of haemoglobin having diameter of 7.2µm. Thickness of RBCs at the thickest point is 2-2.5µm and a minimum thickness in the centre of 0.8-1µm. This structure of RBC allows for free passage of RBCs from sinusoids of spleen.

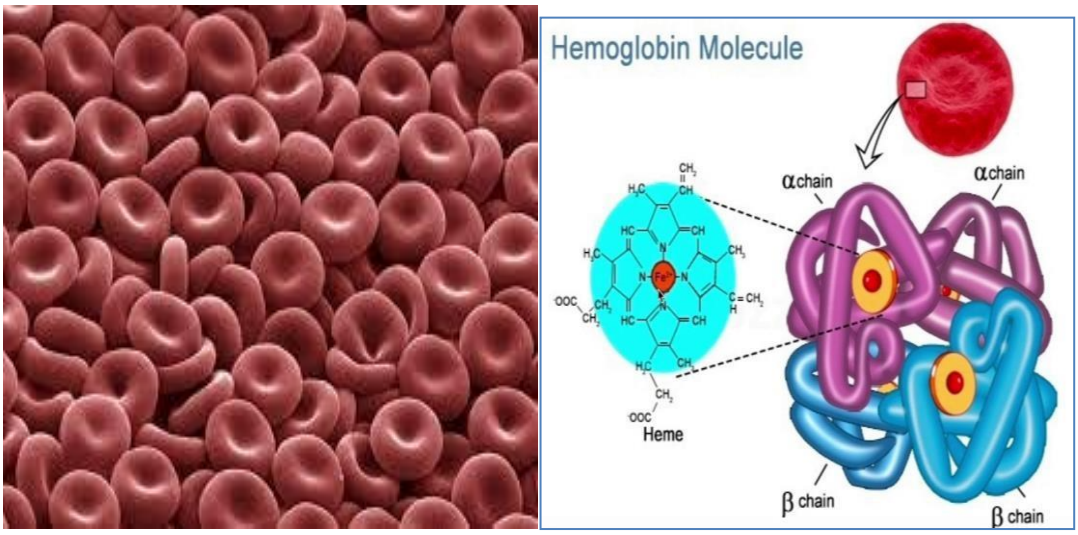


Fig.1.Structure of RBC along with it's composition

Hemoglobin is a tetrameric protein composed of two pairs of globin chains, each with its own heme group. Normal adult red cells contain mainly HbA ($\alpha_2\beta_2$), along with small amounts of HbA_2 ($\alpha_2\delta_2$) and fetal hemoglobin HbF ($\alpha_2\gamma_2$).

Different types of embryonic hemoglobins are:

Gower I: $\zeta_2\varepsilon_2$

Gower II: $\alpha_2\varepsilon_2$

Portland I: $\zeta_2\gamma_2$

Portland II: $\zeta_2\beta_2$

Four levels of highly complex Hb structure:

1. Primary structure: globin chain is a polypeptide that is composed of amino acid sequence[12].
2. Secondary structure: polypeptide globin chains are arranged in α-helixes which is stabilized by hydrogen bonds and separated by non-helical turns[12].
3. Tertiary structure: coiled globin chain arranged in 3-dimensional structure having surface pocket containing haem in between E and F helixes, structure of secondary and tertiary haemoglobin is maintained by binding of this haem in between two specific histidine residues in the E and F helixes[12].(Fig.2)

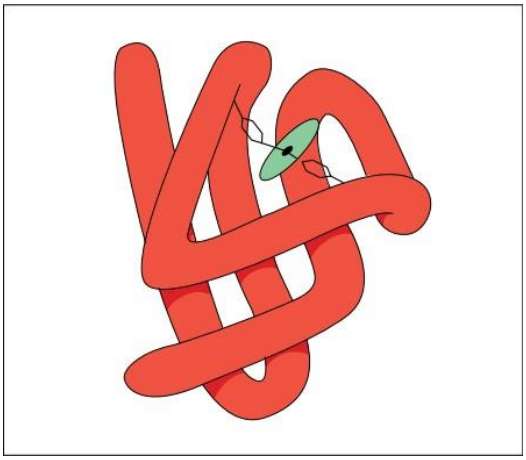


Fig.2.Structure of tertiary haemoglobin having haem pocket containing haem showing binding of haem to proximal and distal histidines of the globin chain while O_2 is bound to distal histidine[12].

4. Quaternary structure: it is made up of 4 globin chains and it is not fixed. Two types of bonds are present, one is dimeric and other is tetrameric, dimericbond

is strong $\alpha_1\beta_1$ and $\alpha_2\beta_2$ bond which holds the molecule in a stable form, while tetrameric bond allows the chains to slide on each other and rotate[12].

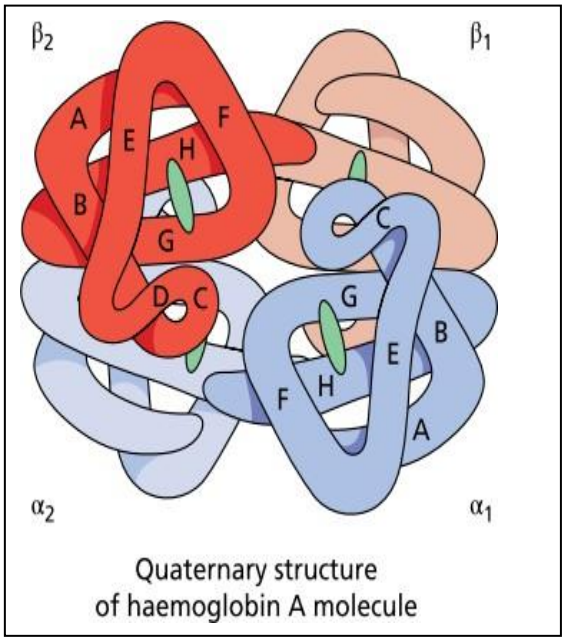


Fig.3.Quaternary structure of hemoglobin[12]

Function of Hemoglobin:

Haemoglobin transports oxygen to the tissues, transports carbon dioxide (CO_2) and also acts as a buffer. Haemoglobin is composed of haem and globin, haem helps in oxygen transport while globin protects haem from oxidation which allows variation in oxygen affinity and makes the molecule soluble[12].

Different types of hemoglobin in different stages of life:

Embryonic hemoglobin will be formed till 8th week of intrauterine life whereas fetal hemoglobin will start forming after 8 weeks of intrauterine life. Main Hb in intrauterine life (i.e. upto 34-36wks of gestation) is HbF (90%) while HbA is around 4-13% of total Hb in the fetus[13]. HbA levels increase to 20-30% and HbF levels decrease to 55-90% of total Hb at term[13].

At 6months of age HbA is 95-96%, HbA_2 is 2-3.5% and HbF<2-3% of Hb[13]. But exact values of different types of Hbs are reliable only after attaining 1yr.

Different stites of hemoglobin synthesis in different stages of life:

In early embryonic life i.e. between 3-8wks of gestation, specific embryonic Hbs are synthesized in the yolk sac. While from 8thwk onwards liver and spleen takeover and they start producing fetal and adult Hbs[12].

In late intrauterine life, bone marrow becomes the major site of Hb synthesis and increased amount of HbA is produced. In adults also, bone marrow erythroblasts predominantly synthesize HbA and other minor Hbs[12].

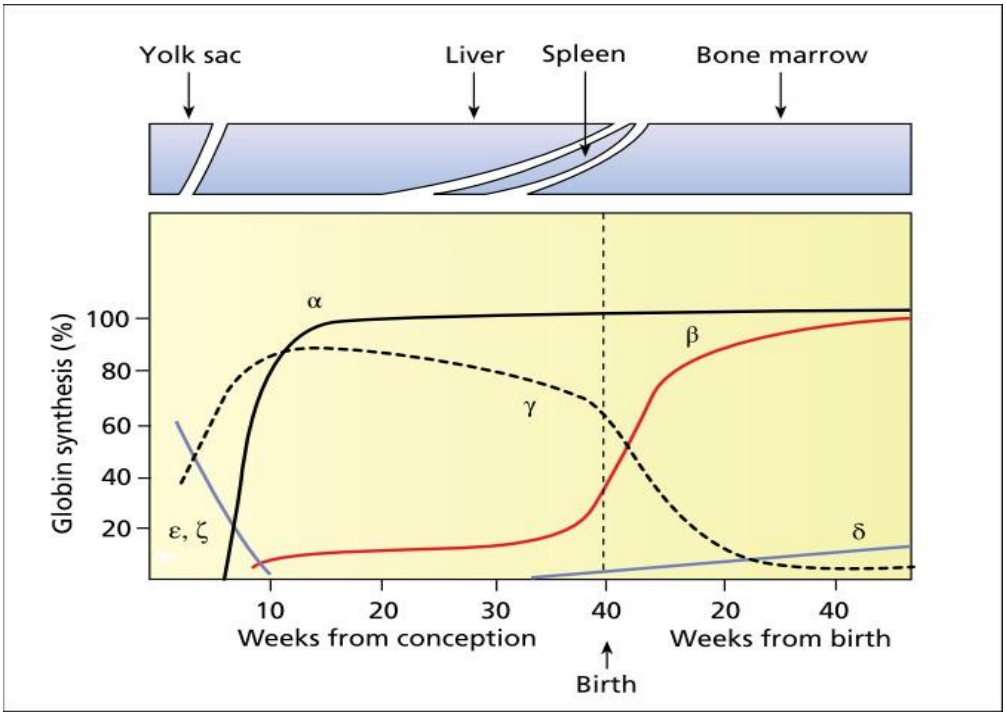
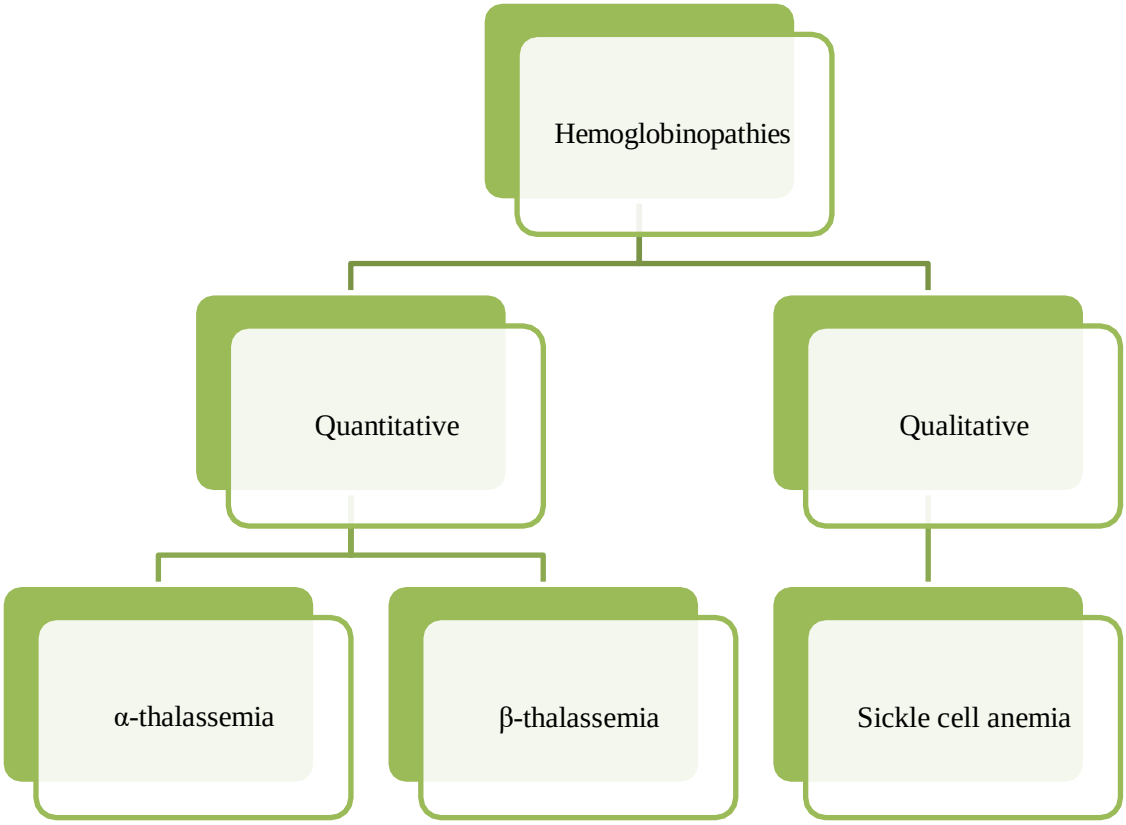


Fig.4.Synthesis of different globin chains in embryo, fetus and infants at different sites and rates[12].

Classification of hemoglobinopathies:



Most common genetic lesion in α-thalassemia is deletion, whereas in β-thalassemia it is non-sense type of point mutation and sickle cell anemia it is missense point mutation.

Thalassemia:

-autosomal recessive

-Most common hemoglobinopathies in World/ India.

α-Thalassemia:

-Decrease in α chain synthesis

-There are two types of α-thalassemias 1 and 2, amongst which α-thalassemia 2 has frequency of 30-50% throughout the World[14].

-α gene mutation present on chromosome 16

-1α globin chain is encoded by 4α genes

-α chain: αα/αα

-types:

- 1α gene deletion: carrier
- 2α gene deletion: trait
- 3α gene deletion: HbH/ β4 tetramers
- 4α gene deletion: BartsHb

-Peripheral smear: microcytic hypochromic anemia; anisopoikilocytosis with target cells.

-Golf ball RBCs is characteristic feature of HbH disease which is demonstrated with the help of Brilliant cresyl blue which is a supravital stain.

β-Thalassemia:

-decrease in β chain synthesis.

-β gene located on chromosome 11.

-Peripheral smear: microcytic hypochromic anemia, anisopoikilocytosis with target cells, pencil shaped cells and Howell Jolly bodies.

-Target cells are most characteristic of thalassemia.

-Screening: most common/ best test in India/ World is NESTROF test (Naked eye single tube RBC osmotic fragility).

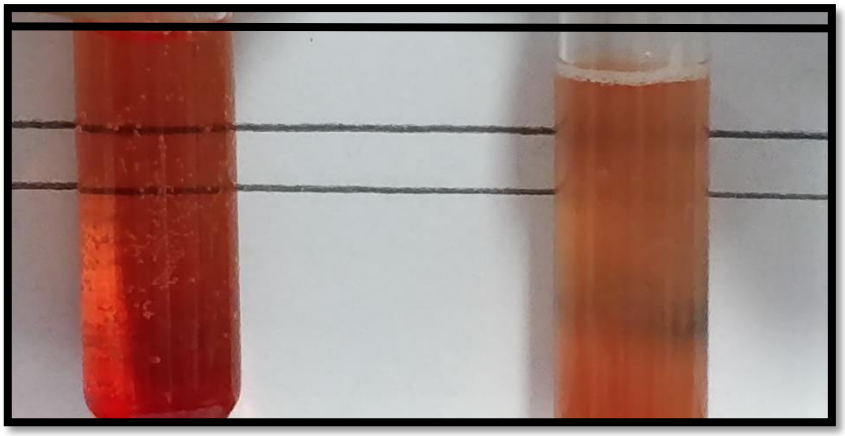


Fig.5 NESTROF test showing positive results

In NESTROF test we add 0.3% NaCl (Normal saline); hemolysis of normal RBCs will be completed by 0.3%NaCl.

No hemolysis in thalassemia at 0.3%NaCl because RBCs membranes are stable.

Chances of high false positive results in our country is expected because there are many cases of iron decifiency in our population. Whereas false negative results are seen in β-thalassemia carriers with NESTROF test, because of which automated analyzers are recommended wherever it is available[13].

Thalassemia major:

-also known as Cooleys/ Mediterranean anemia

-Pediatric (starting from 6-9mon.)

-Hb<3gm%

-Low MCH level in the thalassemia carriers can be compensated by physiologically high RBC (erythrocytosis).

-HbF: 30-90%, HbA_2: high/normal/low

-Most common cause of death is dilated cardiomyopathy

-When both parents are thalassemia minor, then chances of having thalssemia major child is 25%.

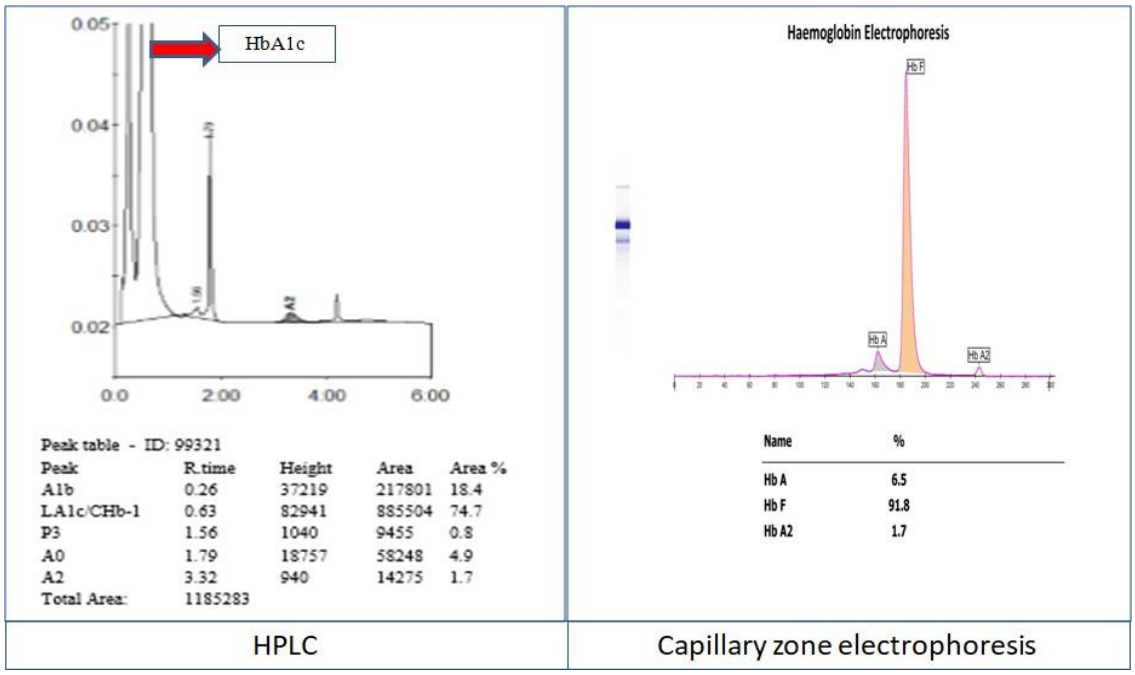


Fig6.Comparision between HPLC and Capillary Zone Electrophoresis in a case of β-thalassemia major

Thalassemia minor/ trait:

-Pediatric/ adult

-Hb: 9-12gm%

-asymptomatic

-no need of blood transfusion

-When both parents are thalassemia minor, then chances of having thalssemia minor child is 50%.

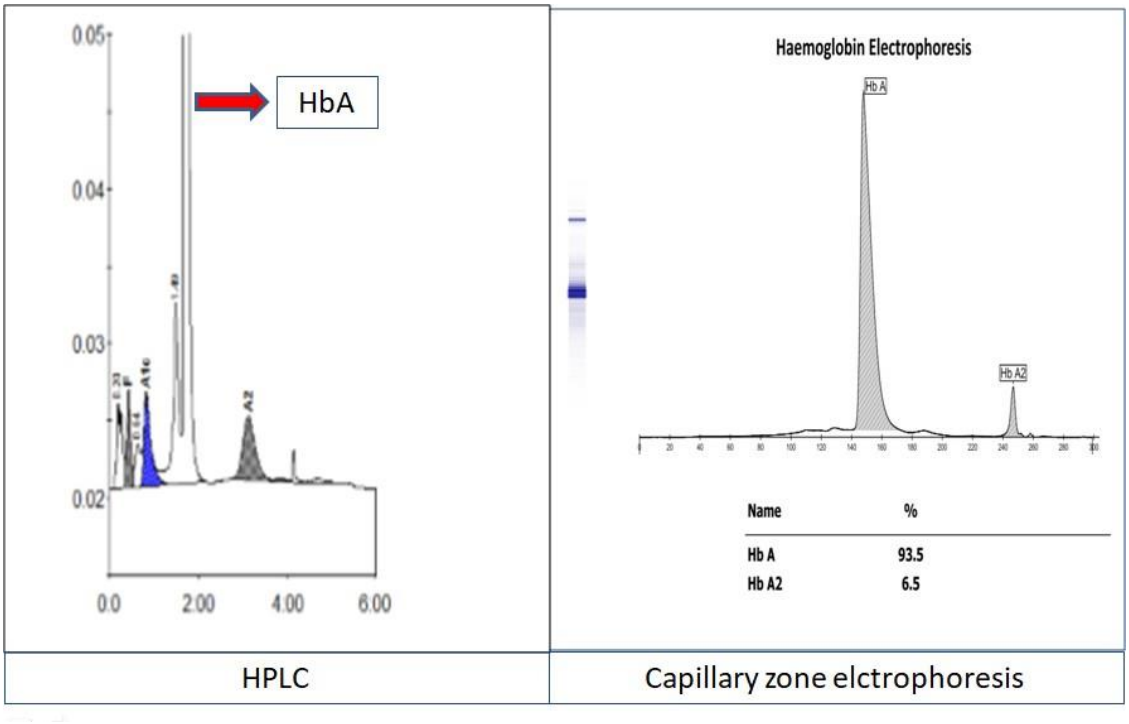


Fig7.Comparision between HPLC and Capillary Zone Electrophoresis in a case of β-thalassemia minor

Thalassemia intermedia:

-Pediatric

-Hb: >7gm%

-Occasionally blood transfusion is required in childhood whereas repeated blood transfusion is required during adolescence.

-HbF: 10-30gm%

Hemoglobin S Thalassemia Syndromes:

The term sickle cell disease includes homozygous HbSS, it also includes all those genotypes which interacts with HbS such as β^{o}-thalassemia, β^{+}-thalassemia, δβ-thalassemia, HPFH and HbLepore. In all the above mentioned heterozygous conditions clinical symptoms are milder than those seen in sickle cell anemia.

a. Hemoglobin S-β-thalassemia:

In this disorder, HbS is most abundant amongst all the Hbs followed by HbA_2 which is <30% in patients with β^+gene mutation whereas it is usually absent in HbS/ β^o-thalassemia. Values of HbA_2 and HbF are increased whereas in some cases value of HbF can be normal[15].

It is clinically similar to sickle cell disease and its severity is directly proportional to the type of β-thalassemia mutation. It includes complications of both i.e. sickle cell disease and thalassemia such as delayed growth and puberty, acute chest syndrome and vaso-occlusive events[15].

These cases are known to undergo avascular necrosis of femoral head. HbS/ β^o-thalassemia patients have high risk of stroke because of which transcranialdoppler screening is advised just like in homozygous HbSS[15].

It is difficult to make out HbS/ β^o-thalassemia and homozygous sickle cell anemia on electrophoresis whereas the scenario is little different in HbS/β^+-thalassemia and sickle cell trait, here on the basis of values of HbS and HbA an accurate diagnosis can be made[15].

It is difficult to evaluate HbA_2 on electrophoresis for a patient of 1yr of age because both the bands of HbA_2 and HbS are in close proximity whereas in HPLC minor components co-elute with HbA_2[15].

Prognosis is anytime better than the individual entities that is Thalassemia major and sickle cell anemia[16].

b. Hemoglobin S-Hereditary Persistence of Fetal Hemoglobin:

In these cases HbF levels are 50-90% during infancy which tends to decrease markedly in the first few yrs of life and stabilizes at around 30% between the age of 3-5yrs[13].

In adults HbS: 70-80%, HbF: 20-30%, HbA_2 decreased and HbA absent, somewhat simulating homozygous sickle cell anemia is seen with electrophoresis whereas HPLC demonstrates HbF>96% and HbA absent in homozygotes and HbF 10-30% in heterozygotes[13].

HbE-Thalassemia:

In these cases gene of β-thalassemia trait is inherited from one parent whereas gene of HbE is inherited from other parent. They have variable clinical and hematological features. Some cases resemble thalassemia intermedia. On peripheral smear microcytic hypochrmic anemia with mild anisopoikilocytosis with few tear drop and target cells are seen, along with it occasional nRBCs are also noted[15].

HbE carriers are frequently seen in the North-East India with a prevalence of 3-64%[13]. Dichlorophenolindolphenol(DCIP) precipitation test is used for screeningof HbE[17].

HbD- Thalassemia:

These cases are result of interaction between HbD and β-thalassemia trait genes. These cases simulate thalassemia intermedia clinically and hematologically. HbD-Punjab is commonly seen in North-Western part of India with a prevalence of 3-4%[13].

Sickle cell anemia:

Autosomal recessive disorder which is common in pediatric age group. HbSS; homozygous- sickle cell anemia

HbSA, heterozygous- sickle cell trait.

Pathogenesis of sickle cell anemia: glutamic acid is replaced by valine in 6th position of β-chain. Glutamic acid is water soluble and negatively charged whereas valine is water in soluble and neutral.

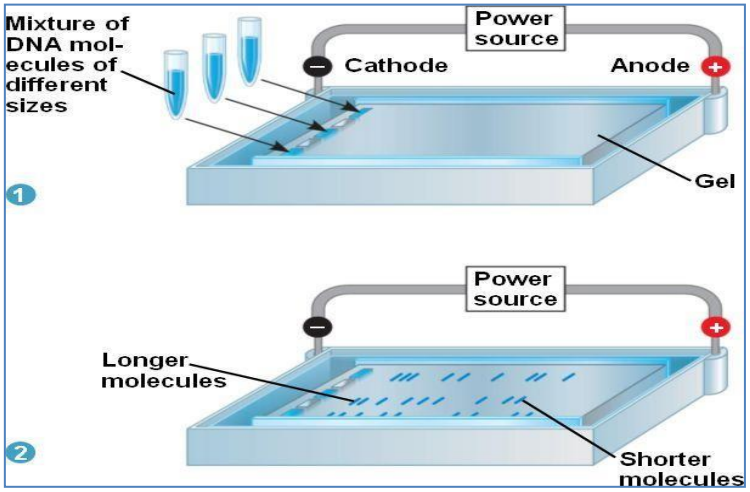


Fig8.Image of gel electrophoresis

HbS molecules stack into polymers when deoxygenated. With continued deoxygenation HbS molecules assemble into long needle-like fibers within red cells, producing a distorted sickle or holly-leaf shape.

Infants do not become symptomatic until they reach 5 or 6 months of age, when the level of HbF normally falls. Molecular testing is required to diagnose a case of sickle cell disease <3mon. of age[13].

Hb variants which copolymerize with HbS are HbC, HbD-Punjab or HbO-Arab and such heterozygous conditions are known as sickle cell syndromes[4].

Clinical features of sickle cell anemia:

1.Vaso-occlusive crisis

- Hand foot mouth syndrome
- Acute chest syndrome
- Sickle cell anemia osteomyelitis
- Autosplenectomy
- Fish – mouth vertebrae

2.Aplastic crisis

3.Sequestration crisis (Medical emergency): most dangerous and life threatening crisis.

Factors affecting sickling:

- ❖ HbS conc.
- ❖ HbSA conc.
- ❖ Hypoxia

Screening:

- ❖ Sodium metabisulphite test
- ❖ Sodium dithionite test

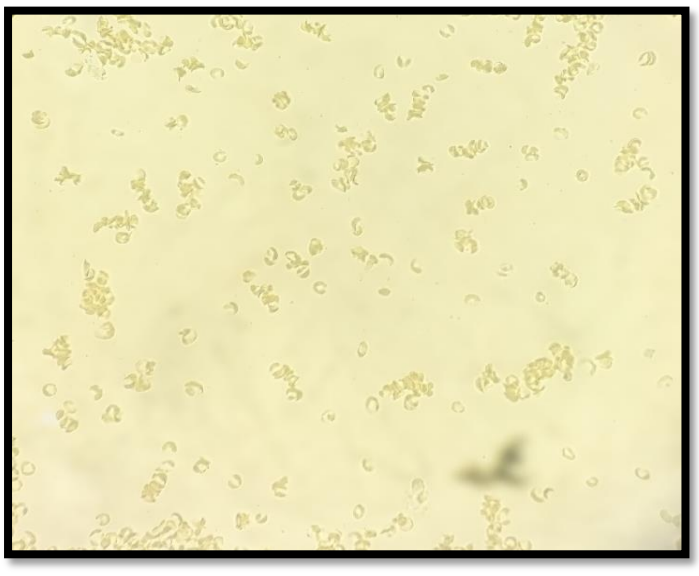


Fig9.Image of sickling test showing sickle/crescent shaped RBCs

Screening for haemoglobinopathies:

Individual groups where screening and diagnosis are indicated:

- Premarriage screening
- Antenatal screening
- Preconception screening
- Neonatal screening
- Preoperative/ preanesthesia screening

Premarriage screening:

It is done in to identify carriers of sickle cell anemia and β-thalassemia. It can be done in schools, colleges or community centers[13].

Antenatal screening:

It is to be done in all pregnant women irrespective of their gestational age[13].

***Preconception screening*:**

In Indian setup it is little difficult for such kind of screening but it can be implemented on those couples who arrive at clinic for assisted conception. In such cases donor's sperm/ova and receiver should be screened for haemoglinopathies[13].

Neonatal screening:

Mainly applicable for sickle cell disease. Suspected babies should be screened either at birth or within the first 4wks after birth with the help of molecular analysis. Penicillin prophylaxis and pneumococcal vaccination should be given to babies with sickle cell disease or sickle β-thalassemia for better outcome or to minimize their morbidity[13].

Prenatal diagnosis:

Chorionic Villus Sampling(CVS) is usually done between 10-12wks of gestation under ultrasound guidance. Then this sample is analysed by mutation

detection methods such as Amplification Refractory Mutation System (ARMS) and Covalent Reverse Dot Blot hybridization (CRDB)[13]. This is not frequently done in India as many pregnant women come to clinicians during her 2nd trimester, for such cases fetal blood sampling by cordocentesis/ amniocenetsis sample is tested[13].

In the above mentioned screening methods if the fetus is detected as thalassemia major then obstetrician is supposed to counsel the couple to go for termination of pregnancy.

Preoperative/ preanesthesia screening:

Recommended only for those ethnic groups which have high prevalence of HbS as it may interfere with preoperative techniques/ clinical management[13].

Reticulocyte count:

Reticulocytes are juvenile or young red cells which appear into the blood stream after getting released from bone marrow and contains ribonucleic acid (RNA) remnants[18].

A reticulocyte count is indicated if a blood film shows polychromasia or if haemoglobin H disease or an unstablehaemoglobin is suspected[12]. A supravital dye like Brilliant Cresyl/Methylene Blue can be used to analyze reticulocyte count in which remnants of RNA are stained as blue precipitating granules or filaments[18]. Argon laser based flow cytometer can also be used to calculate reticulocyte count in which Auramine O (a fluorescent dye) is used to stain reticulocytes followed by passing it through a laser beam[15].

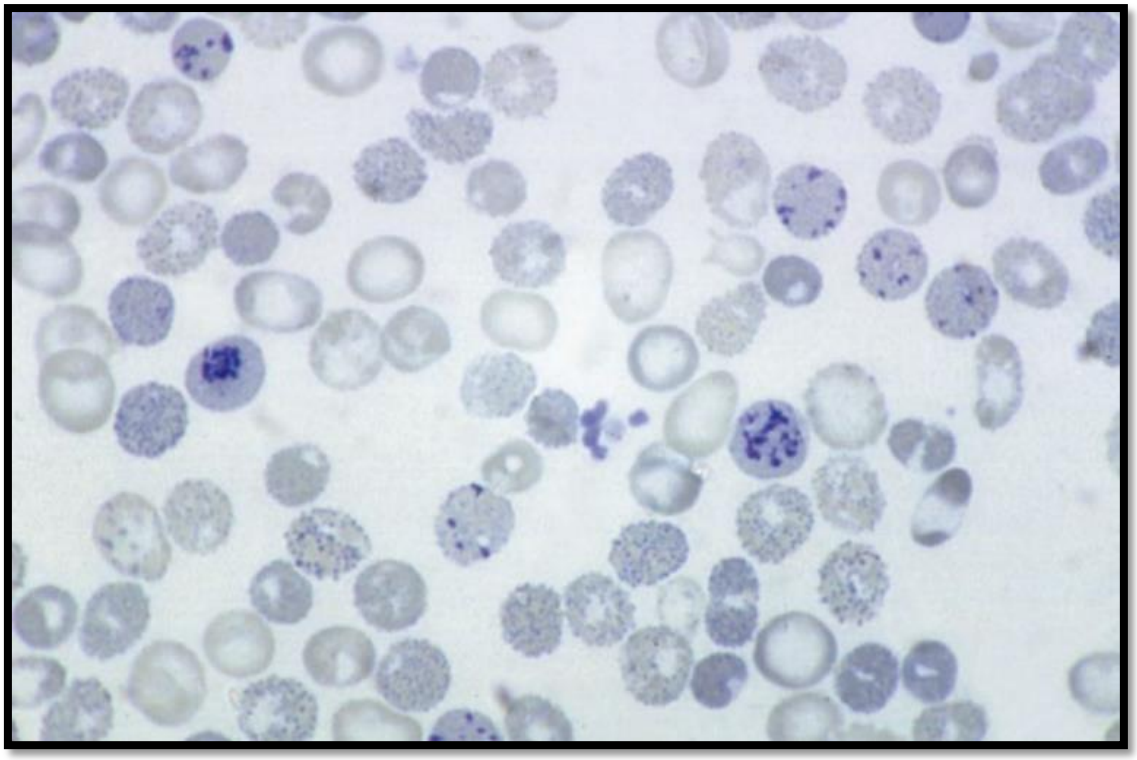


Fig10. Reticulocyte preparation in a β-thalassemia major patient[12].

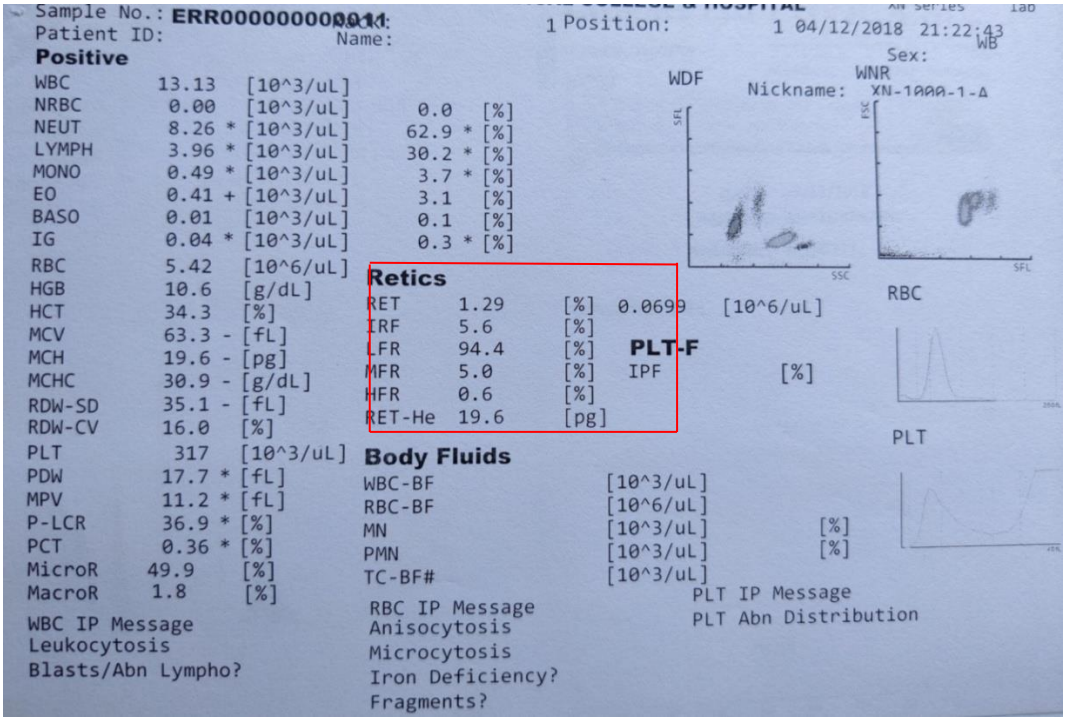


Fig11. A cell counter report showing reticulocyte count

This report is showing absolute number and % of reticulocytes, IRF(Immature reticulocyte fraction), LFR(Low fluorescence reticulocytes), MFR(Medium fluorescence reticulocytes) and HFR(High fluorescence reticulocytes). LFR and MFR tells us about the % of reticulocytes while HFR tells about % of newly formed (immature) reticulocytes[15]. New cell counters are useful to analyze response to iron therapy (in 1-2 days) in IDA and in a case on EPO therapy.

Modalities used for diagnosing different haemoglobinopathies

1.HPLC

It can diagnose hemoglobinopathies on the basis of proportion of hemoglobin variants and retention time[19].

Retention time is the time required by HPLC machine for gradient elution of different Hb fractions. It is the duration between sample injection and maximum point of each peak[20]. It helps to identify peaks of different hemoglobins[20]. Common retention time will be seen for many unknown and known variants of Hb; because of which all such Hb variants will elute in the same window[20].

HPLC gives an accurate estimate of HbA_2 levels and is the method of choice[13]. HPLC has excellent resolution, quantification and reproducibility of many normal and abnormal haemoglobins[9].

HPLC cannot measure exact HbA_2 value in HbE thalassemia cases[21]. Whereas it can exactly measure HbD values which CZE cannot.

Limitations of HPLC are: knowledge, experience and skill is required along with costly setup[19].

Fig.12.HPLC Bio-Rad D-10

2.Hemoglobin electrophoresis

Hemoglobin electrophoresis can be carried out on various mediums such as cellulose acetate membrane, filter paper, a starch gel, a citrate agar gel or an agarose gel. But the best amongst them for hemoglobin electrophoresis is lysed packed red cells which cause uniform application of consistent amount of haemoglobin and removes unwanted plasma proteins which causeshinderance in the outcome[12].

Types of hemoglobin electrophoresis:

a. Cellulose acetate electrophoresis
b. Agarose gel electrophoresis
c. Capillary electrophoresis

a.Cellulose acetate electrophoresis

In this method of analysis freshly prepared hemolysates are used on cellulose acetate membrane at alkaline pH followed by elution of bands to quantify HbA_2. It requires experienced technicians, it is time consuming and cumbersome[13].

It permits evaluation of HbA,F,S/G/D/Lepore, C/E/O-Arab and few less common variant such as HbQ-India, HbD-Punjab and HbD-Iran [12].

Earlier this method was used for neonatal screening for quantification of HbS frequently as it is cost effective. It helps to differentiate between sickle cell trait and β thalassemia by accurately measuring the percentage of HbS. It is also used to determine the outcome of exchange transfusion in sickle cell anemia patients by exactly evaluating percentage of HbS[12].

b.Agarose gel electrophoresis

It is more expensive and less convenient than cellulose acetate electrophoresis and is little used[12].

c.Isoelectric focusing(IEF)

It is a type of gel electrophoresis in which commercially available plates of cellulose acetate containing carrier amphoteric molecules of various pIs/ polyacrylamide are used which creates a pH gradient. Here, isoelectric point (pI) is the point at which no net charge is produced. The Hb molecules migrate along the plate to reach a point at which pH corresponds to the pI of Hb when the plate is coated with haemolysate and kept in a strong electric field. After that densitometry is used to quantify various stained Hb bands[12].

Bands which are obtained by IEF are much more sharper than cellulose acetate electrophoresis. Cellulose acetate electrophoresis is much cheaper than IEF. In IEF glycosylated Hb and methamoglobin resolves separately which makes interpretation difficult[12].

Capillary Zone Electrophoresis (CZE)

In 2007 Food and Drug Administration approved sebia capillary system (Sebia, Norcross,GA) for diagnosing hemoglobinopathies[21].

Electrolyte buffer-filled silica capillary is used for electrokinetic separation of molecules. Capillaries with diameter of <100µm are filled with positively charged electrolyte buffer which is adjusted at a very high temperature and voltage which helps us to do eight simultaneous analysis[22].

It is used for detection of monoclonal gammopathies and other protein abnormalties in serum[21]. It is fully automated[22]. It can separate and quantitate HbA_2, HbE, HbF, HbH and Hb Bart's[14]. Less turn around time, high throughput, minimal sample manipulation, less volume of sample is required and cost effective[14]. It can measure exact value of HbA_2 in HbE thalassemia[21]. Also used to quantify Hb Bart's[22].

Fig12.Sebia minicap-2 flex piercing (capillary zone electrophoresis)

MATERIALS AND METHODS

Source of data :

A prospective hospital based study was conducted in the department of Pathology(Central laboratory), BLDEU'S Shri B.M.Patil Medical College, Hospital and Research centre, Vijayapura.

Study period: 1st December 2018 to 30th May 2020

Inclusion criteria:

- Children and adults requiring frequent blood transfusion.
- MCV <72fL and MCH<27pg.
- Clinically suspected cases of hemoglobinopathy or β- thalassemia.
- Relatives of the cases**.**

Exclusion Criteria

- Patients who had undergone blood transfusion within a span of 12weeks.

Methods of collection of data.

The study included transfusion requiring children and adults along with their relatives excluding those patients who had undergone blood transfusion within a span of 12weeks. Written consent was taken from all patients for using their sample for diagnostic purpose. Data of children and adults requiring blood transfusion was collected like demographic data (age, sex, etc), presenting complaints, number of blood transfusions till date, age at first transfusion and family history was noted.

Blood samples were collected in EDTA anticoagulated vials and were analysed in automated cell counter (Sysmex XN-1000) for complete blood counts. Peripheral smears were prepared and stained using Leishman's stain.

All samples were run on HPLC;Biorad D-10 which is an automated cation exchange HPLC instrument that has been used to quantify HbA_2, HbF, HbA along with screening hemoglobin variants like HbS, HbD, HbE and HbC. Sebia capillary zone electrophoresis was also used to determine various haemoglobinopathies.2 % sodium meta bisulphite was used wherever required to confirm the presence of HbS.

High Pressure Liquid Chromatography:

Cation exchange high pressure liquid chromatography was used to determine Hb variants. Retention time of unknown Hb variants were compared with 4 standard Hbs: HbF, HbA, HbS and HbC. HPLC column was injected with samples collected in EDTA bulb. Readings were then noted, taking into consideration following normal values of adults; HbA:97-99%, HbF: <0.8%, HbA_2: 2.5-3.5%.

Capillary Zone Electrophoresis:

Sebia capillary system was used for electrophoresis. Initially sample was centrifuged for 5000rpm for 5min. followed by segregation of plasma and RBC pellet. As the instrument is totally automated, right from bar code reading till the production of electrophoretogram no technical intervention was needed. Alkaline buffer (pH 9.4) was required during electrophoresis and at 415nm wavelength Hbs were measured. Normal values of adults as recommended by manufacturer's ;HbA: ≥96.8%, HbF: <0.5%, HbA_2: 2.2-3.2%.

Capillary electrophoresis has negative-charged narrow diameter silica capillaries to separate Hb variants under high voltage[22]. It separates normal and abnormal Hb fractions at a high degree of confidence on the basis of pre-determined calibrated zones[22].

Sample Size :

As in the study done by Dogaru M. et al[1], considering the utility of HPLC and Hb electrophoresis in characterisation of hemoglobin profile in hemoglobinopathies with finite population correction, a minimum of 35 subjects were taken as the sample size taken into consideration confidence level to be 95% and margin of error to be ±15%.

Hence the formula used is:

$$n = \frac{Z^2P(1-P)}{D^2}$$

n= sample size

Z = Z statistic for a 15% level of confidence

P = expected incidence

D =desired precision

Statistical analysis:

Data is analyzed using

1. Mean +/- S.D
2. Chi square test for association
3. Comparison of means using test
4. ANOVA for comparison between and among groups
5. Diagrammatic presentation
6. Sensitivity and specificity analysis

RESULTS

The study included transfusion requiring children and adults along with their relatives excluding those patients who had undergone blood transfusion within a span of 12weeks. Study included 41 cases out which most cases(71%) were between the age group of 20-35yrs followed by 17% cases of 2-20yrs and 12% cases of <2yrs.

Table 1. Age wise distribution of cases:

Age (yrs)	No.of cases	% of total case
<2	5	12
>2-20	7	17
20-35	29	71

Fig14. Pie chart showing age wise distribution of cases:

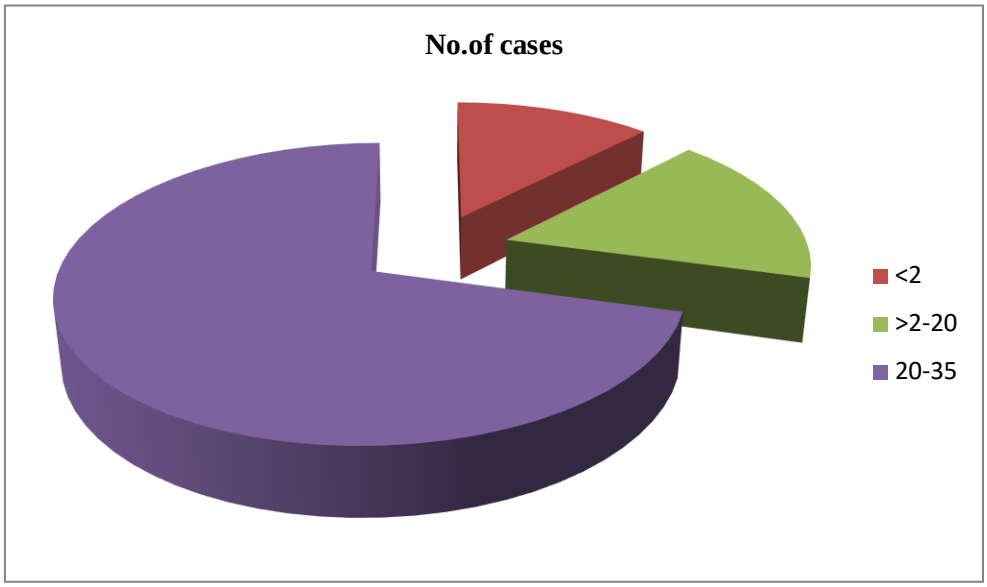


Table2:Laboratory investigations

	n	RBC count		Hb		MCV		MCH		Reticulocyte count	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1.Thalassemia trait	24	570708.3	89911.3	10.9	1.8	62.7	4.1	19.3	2.1	1.2	0.4
2.Normal	9	468375.0	81713.7	11.7	3.3	80.0	11.8	24.9	5.0	1.3	0.8
3.β-thalassemia major	3	262666.7	57239.3	5.7	1.4	68.6	1.3	21.6	0.7	4.3	0.9
4.Homozygous/ double heterozygous for β-thalassemia and δβ- thalassemia	2	176000.0	9899.5	3.9	0.9	69.4	17.8	21.8	4.0	4.6	4.5
5.Sickle cell anemia	1	242000.0	0.0	7.5	0.0	87.2	0.0	31.0	0.0	4.1	0.0
6.Compound heterozygous sickle cell anemia and β-thalassemia	1	431000.0	0.0	9.3	0.0	64.5	0.0	21.6	0.0	4.1	0.0
7.Compound heterozygous for HbE/ β-thalassemia	1	363000.0	0.0	7.4	0.0	63.1	0.0	20.4	0.0	2.9	0.0
Total	41	481561.0	155224.0	10.0	3.1	67.7	9.9	21.3	4.1	1.8	1.5
p value		<0.001*		<0.001*		<0.001*		<0.001*		<0.001*	

*means Sig ($p<0.05$)

Mean RBC count for β-thalassemia trait is more than the upper limit of normal range whereas that of β-thalassemia major is there within the normal range. Mean Hb concentration of β-thalassemia trait is towards lower limit while compared to other hemoglobinopathies which showed significant decrease in Hb concentration. Mean MCV and MCH is decreased in all cases except for those in which we got normal impression and in a case of sickle cell anemia where MCV as within normal limit. Except for β-thalassemia trait and normal cases all other cases showed increase in observed reticulocyte count (Normal observed reticulocyte count=0.5-2.5%).

Table 3.Different hemoglobinopathies obtained using hemoglobin electrophoresis and HPLC:

	No.of cases	% of total case
1.Thalassemia trait	24	58.5%
2.Normal	9	22.3%
3.β-thalassemia major	3	7.2%
4.Homozygous/ double heterozygous for β-thalassemia and δβ- thalassemia	2	4.8%
5.Sickle cell anemia	1	2.4%
6.Compound heterozygous sickle cell anemia and β-thalassemia	1	2.4%
7.Compound heterozygous for HbE/ β-thalassemia	1	2.4%

In our study maximum no. of cases(24) were of thalassemia trait, 9 cases were normal, 3 cases of β-thalassemia major, 2 cases of homozygous/ double heterozygous for β-thalassemia and δβ-thalassemia, 1 case of sickle cell anemia, 1 case of Compound heterozygous sickle cell anemia and β-thalassemia and 1 case of compound heterozygous for HbE/ β-thalassemia.

Fig15.Bar diagram showing different hemoglobinopathies obtained by using electrophoresis and HPLC:

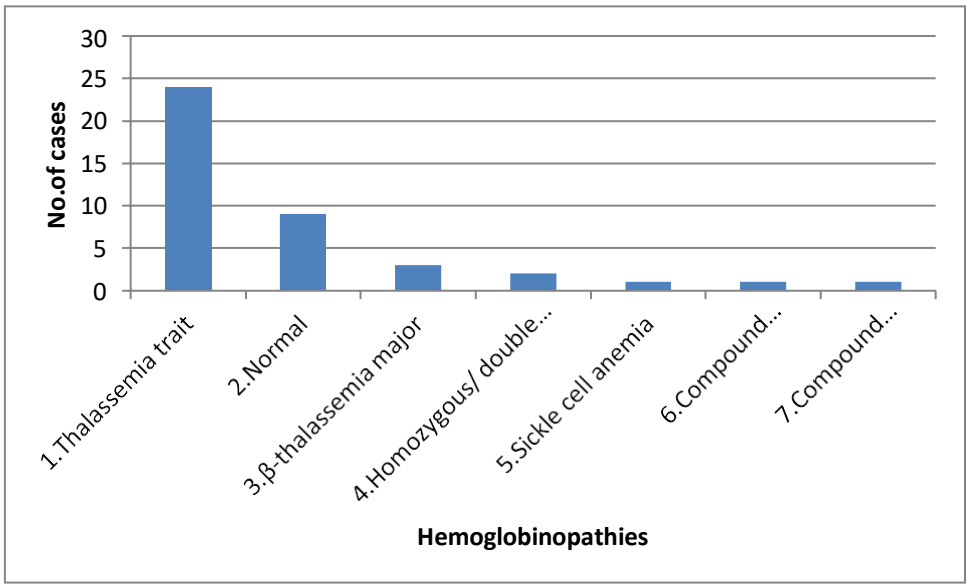


Table4: Comparision of HbF values between HPLC and Electrophoresis in adults(n=36)

	HPLC	Electrophoresis
<0.8%	18	3
≥0.8%	14	7
ND	4	26
Total	36	36

Chi square=29.181

P<0.0001;which means there is significant difference in the values of HbF obtained by both the modalities. This huge difference is because electrophoresis cannot determine efficiently HbF value when it is <0.8 whereas HPLC can do that.

Fig16.Bar diagram showing comparision of HbF values between HPLC and Electrophoresis in adults(n=36):

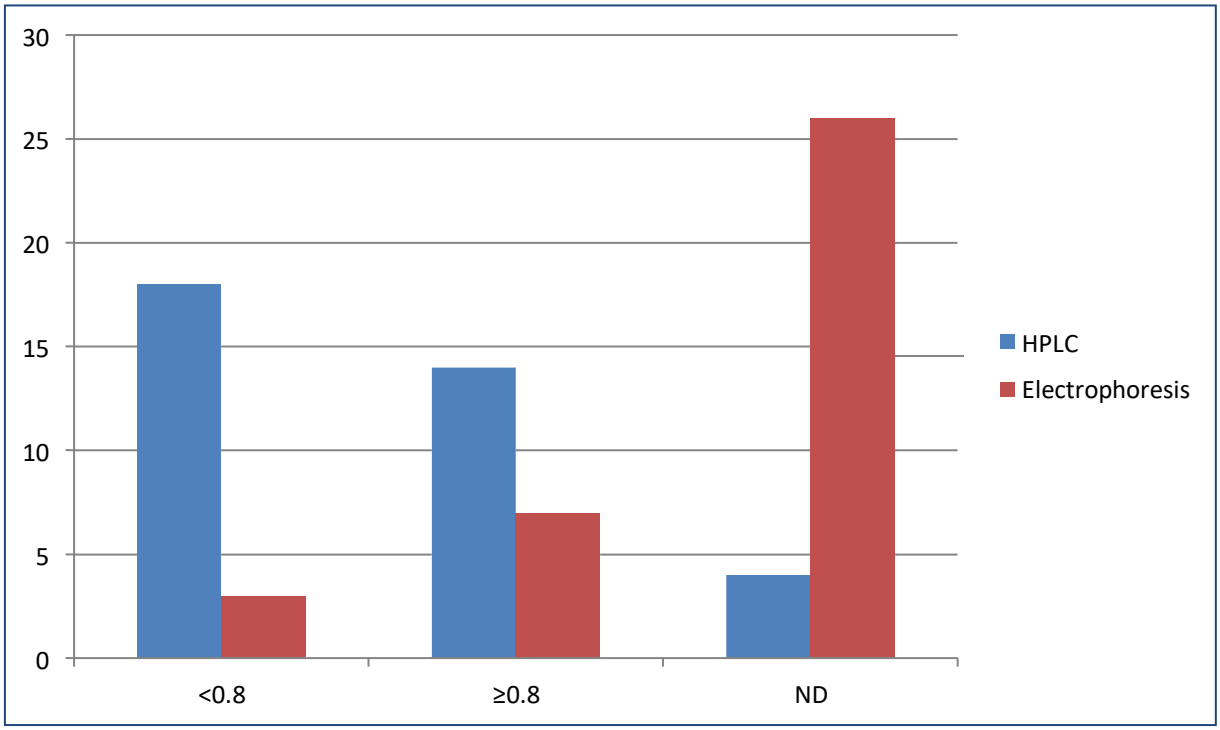


Table5. Comparision of HbF values between HPLC and Electrophoresis in children <2yrs of age (n=5):

	HPLC	Electrophoresis
3-15%	1	1
≥15%	3	3
ND	1	1
Total	5	5

Chi square=0.00

P=1,No significant difference in the HbF values in children <2yrs of age obtained by both the modalities is noted.

Fig17.Bar diagram showing comparision of HbF values between HPLC and Electrophoresis in children <2yrs of age (n=5):

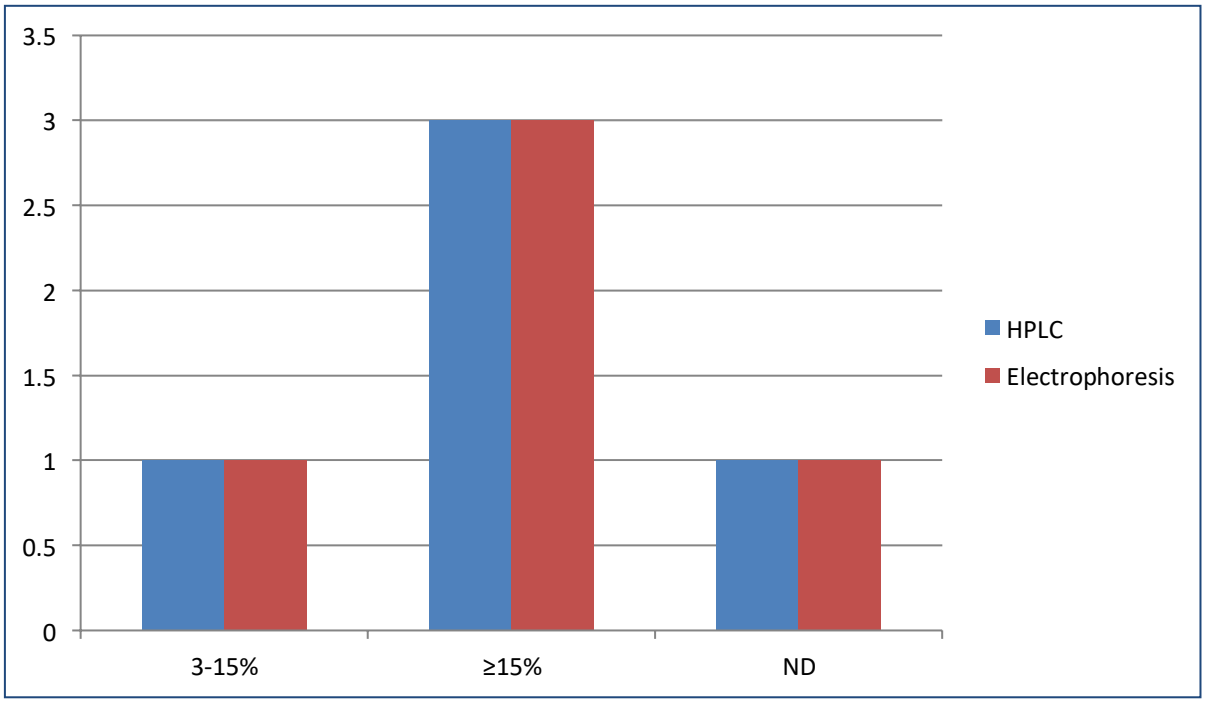


Table6. Comparision of HbA_2 values between HPLC and Electrophoresis(n=41):

	HPLC	Electrophoresis
<3.5%	15	15
≥3.5%	26	26
Total	41	41

Chi square=0.00

P=1,No significant difference in the HbA_2 values obtained by both the modalities is noted.

Fig18. Bar diagram showing comparision of HbA_2 values between HPLC and Electrophoresis(n=41):

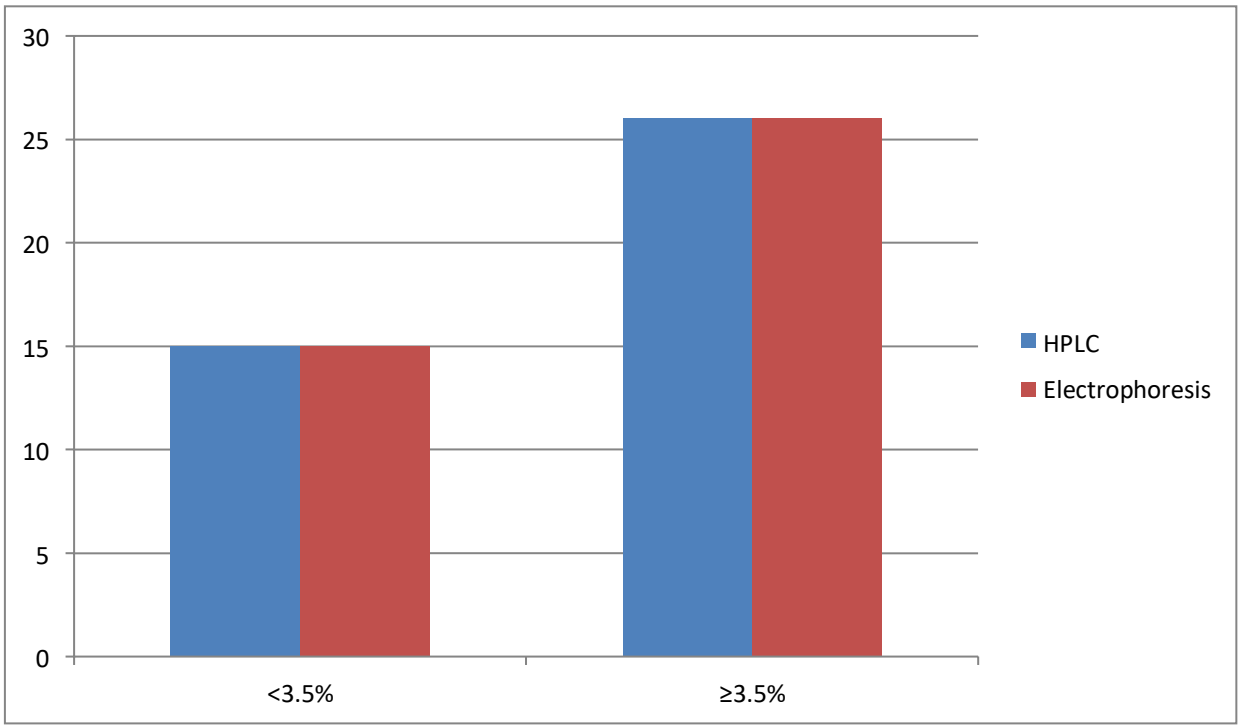


Table7: Mean and SD of different Hb variants obtained by both the modalities i.e. HPLC and Electrophoresis

		HPLC	Electrophoresis
HbF	Mean±SD	26.07±35.22	30.66±37.71
HbA_2	Mean±SD	4.19±1.67	4.03±1.64
HbA	Mean±SD	81.08±30.84	82.03±30.64

HbF

Mann –Whitney U test=120.0

P=0.8345, No significant difference is noted in both the modalities in evaluating HbF values.

HbA_2

Mann –Whitney U test=761.0

P=0.4637, No significant difference is noted in both the modalities in evaluating HbA_2 values.

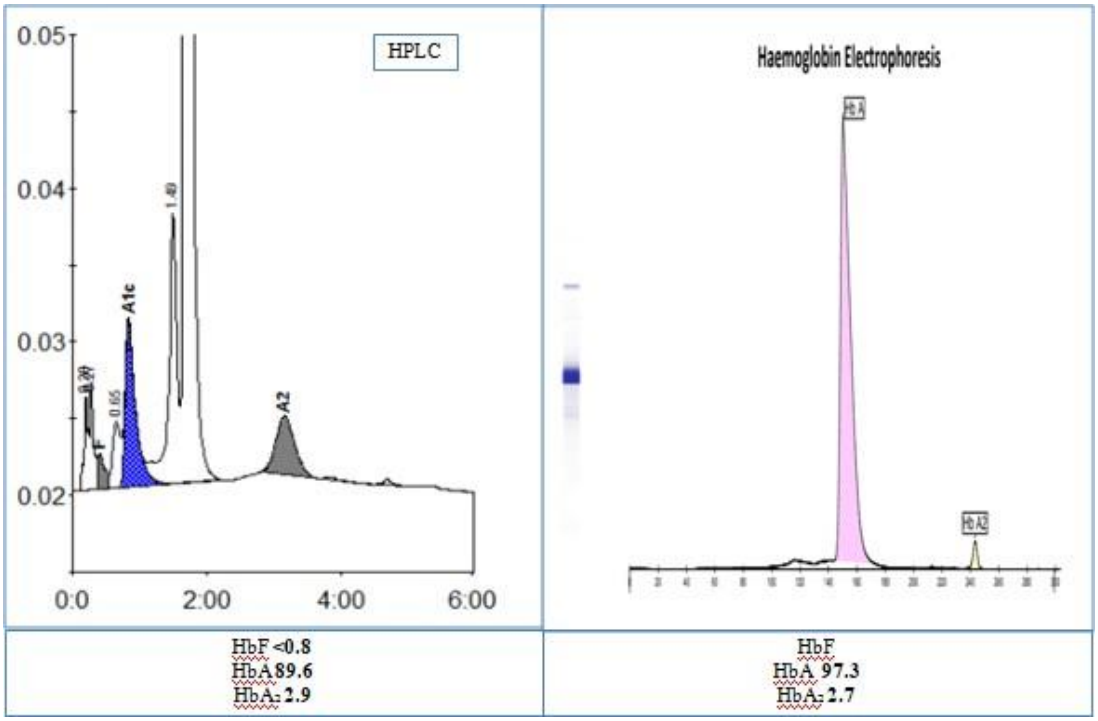
HbA

Unpaired t test=0.1399

P=0.8891, No significant difference is noted in both the modalities in evaluating HbA values.

Variation is not statistically significant in evaluating HbF, HbA_2 and HbA by using both the modalities i.e. HPLC and CZE.

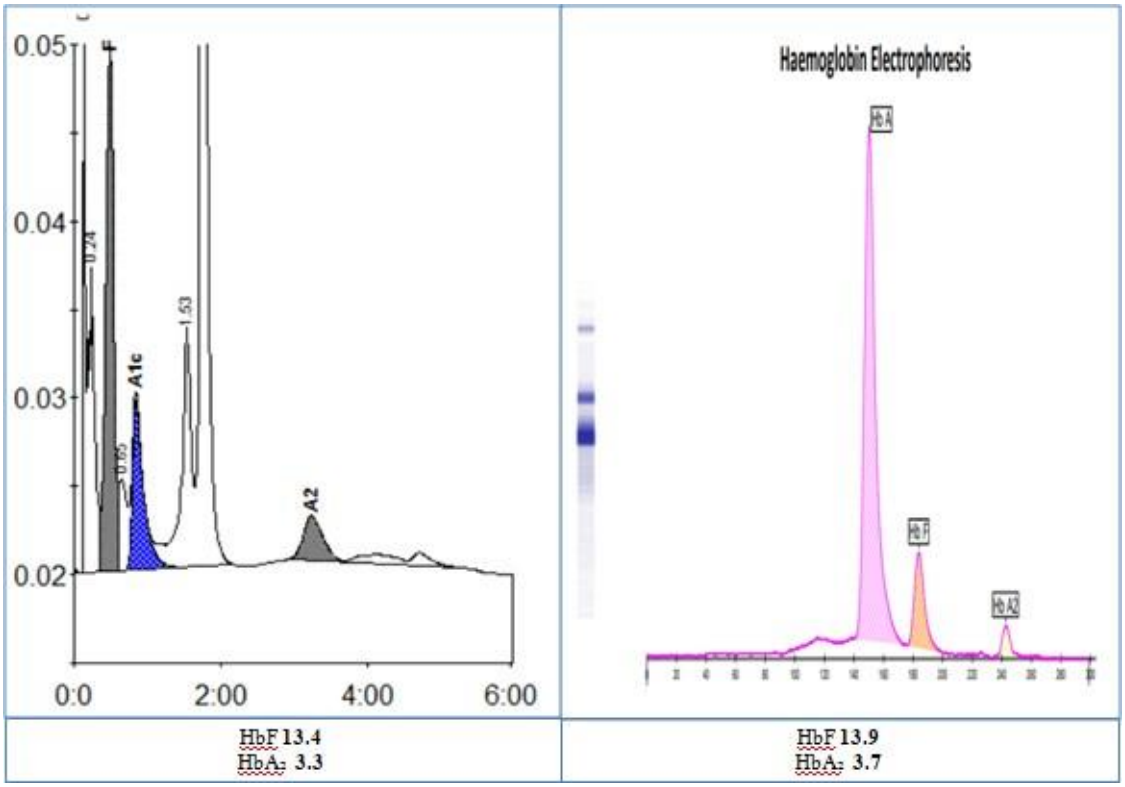
Fig19.Comparision of HPLC and Electrophoresis in a case of β-thalassemia trait:



Twentyfour cases of β-thalassemia trait gave same results in both HPLC as well as capillary zone electrophoresis with a very minute difference in the value of HbA_2 i.e. HbA_2 levels are slightly higher in CZE than that of HPLC[22].Normal HbF values in adult is <0.8% and HbA_2 is <3.5%. And if HbA_2 is between 3.5-3.9% then it is suspicious of β-thalassemia trait. Maximum value of 8-9% of HbF is acceptable in cases of β-thalassemia trait[23].

The low MCV of β-thalassemia carriers can be masked by the larger size of the HbF cells in cases of β-thalassemia trait associated with elevated HbF[23]. Hb concentration in β-thalassemia trait is usually towards lower limit of normal range because of which those patients remain asymptomatic till adulthood and are diagnosed while doing screening.

Fig20.Comparision of HPLC and Electrophoresis in case no.33:

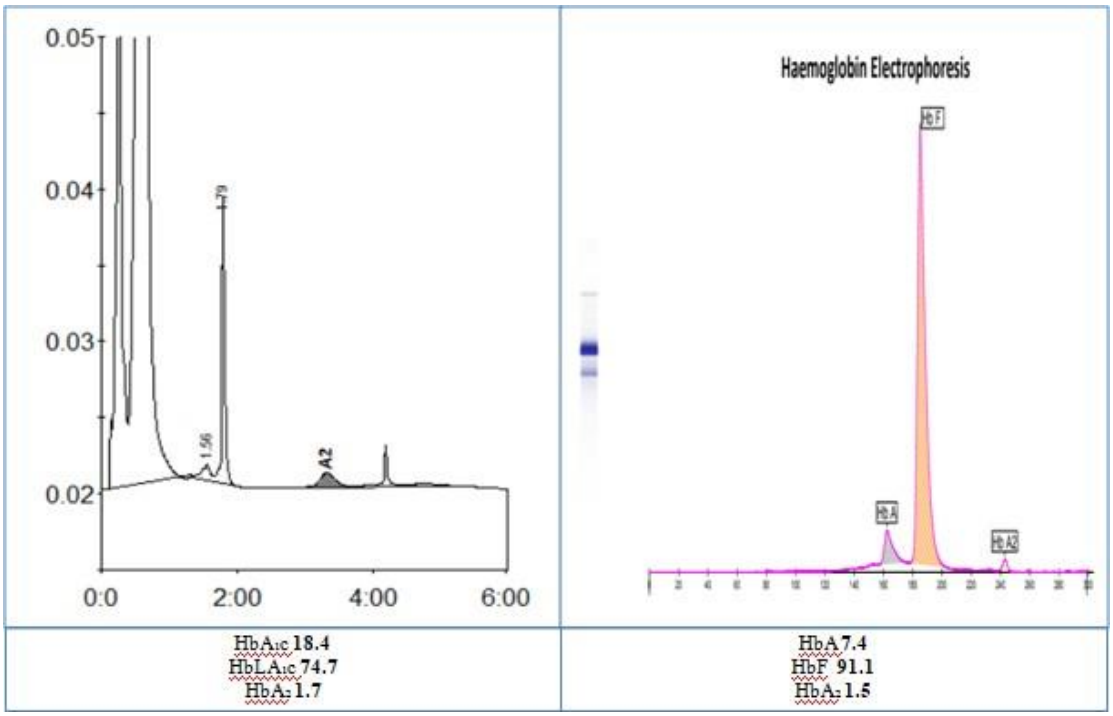


In this case serum ferritin assay was advised keeping in mind suspicion of IDA. The parameter which can distinguish between IDA and thalassemia is red blood cell count[23].

In cases with both β-thalassemia trait and IDA, levels of HbA_2 will not come to normal because of iron deficiency[23]. Few studies have shown no significant difference in HbA_2 levels in patients having both IDA and β-thalassemia trait[24,25].To differentiate between IDA and β-thalassemia trait many algorithms based on erythrocyte indices were figured out[26]. One of those indices is Mentzer's index which came out to be >13 in this case.

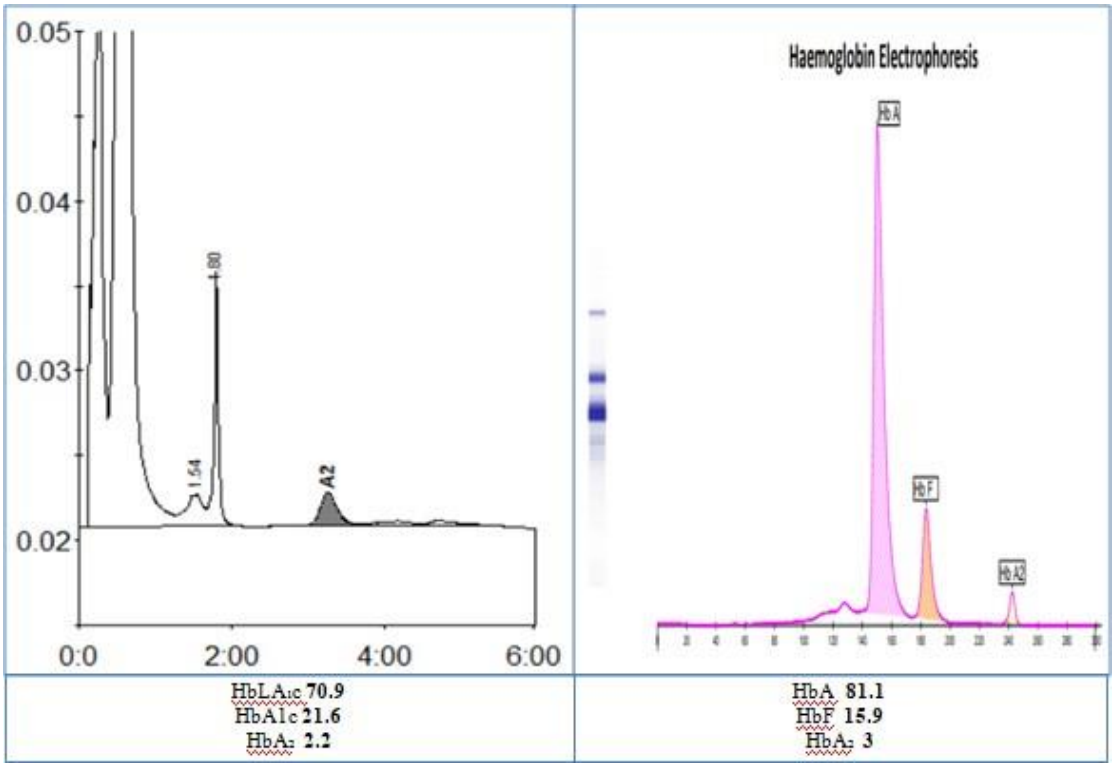
Family study plays a very important role in such cases[27].

Fig21.Comparision of HPLC and Electrophoresis in a case of β-thalassemia major:



In our machine i.e. BIO-RAD, D-10 dual program(extended) whenever HbF value is >16.5% it goes and elutes with $HbLA_1c$ and it actually cannot interpret beyond its normal range i.e.0.8-16.5. So in such cases wherever we required exact value of HbF to make out whether it is thalassemia major or thalassemia intermedia Capillary zone electrophoresis was done additionally. Capillary zone electrophoresis gave exact value of HbF on the basis of which we can make out whether it is β-thalassemia intermedia or major as HbF for intermedia is 10-30% where as for major it is 30-90%,

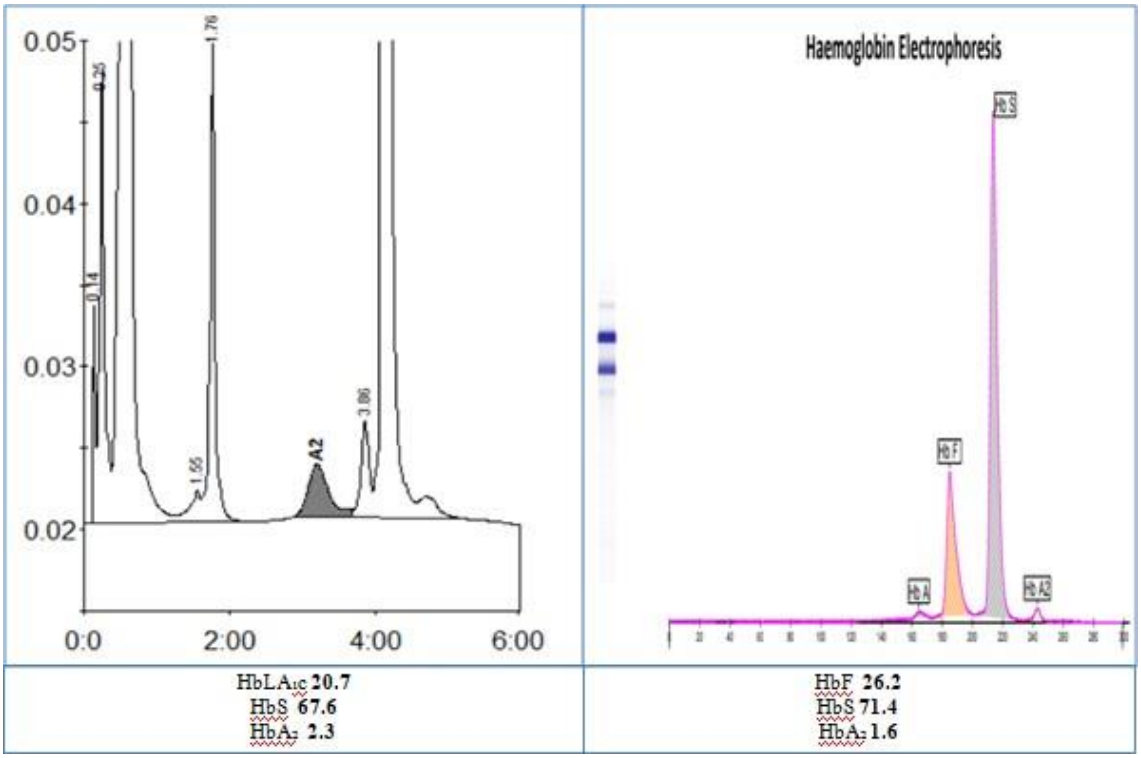
Fig22. Comparision of HPLC and Electrophoresis in a case of Homozygous/ double heterozygous for β-thalassemia and δβ-thalassemia:



It is a heterozygous condition in which there is increased synthesis of HbF and decreased/ absent synthesis of HbA[28]. Two such cases were diagnosed, both of them showed raised HbF during early infancy which raised the suspicion of δβ-thalassemia or HPFH (Hereditary Persistence of Fetal Hemoglobin)[22].

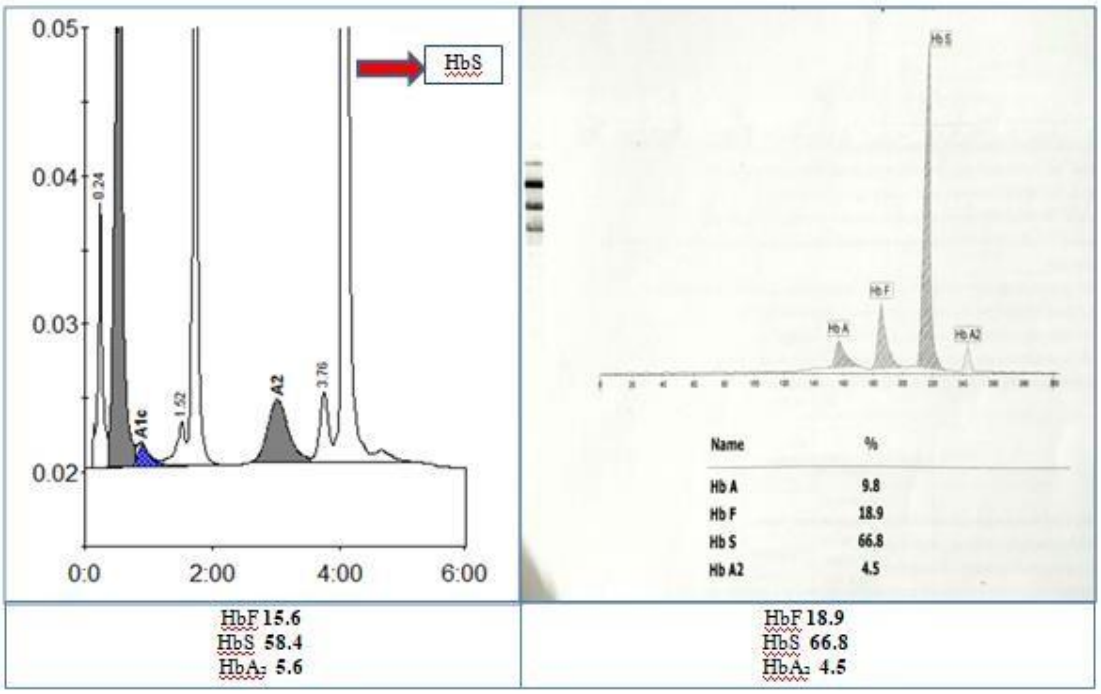
DNA analysis is required to differentiate between HPFH and compound heterozygous δβ-thalassemia which appears same on HPLC and CZE[28]. Double heterozygous for β-thalassemia and δβ-thalassemia has severe outcome whereas HPFH is asymptomatic because of which their distinction is very important[28].

Fig23. Comparision of HPLC and Electrophoresis in a case of Sickle cell anemia:



One case of sickle cell anemia was diagnosed in our study in which the value of HbS was more in CZE whereas value of HbA_2 detected was more in HPLC. In this case exact value of HbF was not detected in HPLC because it got eluted with $HbLA_1c$. The values of CBC for this case was not deranged but if it was associated with α-thalassemia then it would had deranged CBC values[23]. Molecular analysis is not required for confirming the value of HbS[23].

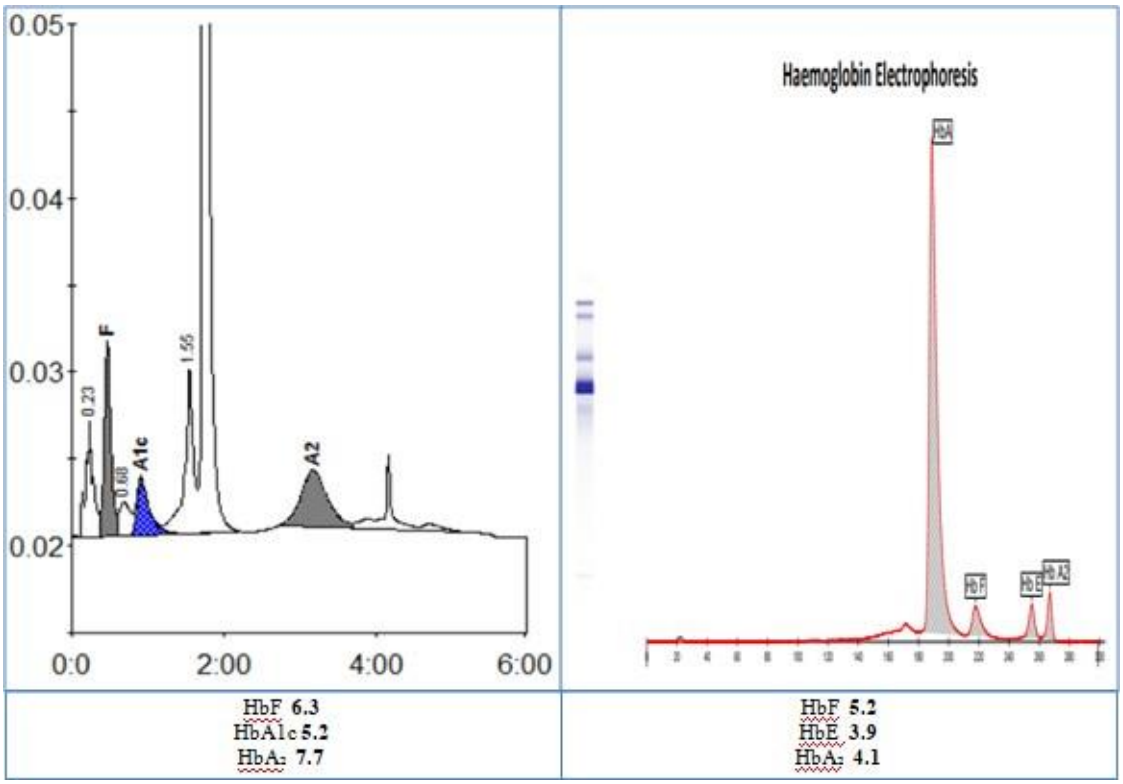
Fig24.Comparision of HPLC and Electrophoresis in a case of Compound heterozygous sickle cell anemia and β-thalassemia:



Whether it is sickle cell anemia or sickle β-thalassemia HbS values are high when evaluated by CZE as compared to HPLC, but both the modalities gave similar data to make a final diagnosis. Falsely elevated HbA_2 was recorded while doing screening for Hb variants (HbS, HbE) by various authors[30].

A reference value of 3.5% for HbA_2 should not be used to diagnose sickle cell anemia, when HbA_2 is evaluated by HPLC.

Fig25.Comparision of HPLC and Electrophoresis in a case of Compound heterozygous for HbE/β-thalassemia:



HbE thalassemia is most common in North-Eastern part of the country. It has a very severe outcome. HPLC is unable to detect HbE value because of which whenever HbA_2 values in HPLC is >10% then one should suspect presence of Hb variants such as HbD/ HbE and should go for complementary diagnostic modality like CZE which will exactly detect HbE like we got in this case.

Table8.Values of Hb variants using HPLC and electrophoresis in 8 unusual cases:

	HPLC			Electrophoresis		
	HbF	HbA2	Others	HbF	HbA2	Others
1.β thalassemia major1	91.2	1.2		91.1	1.5	
2.β thalassemia major2	93.1	1.7		91.8	1.7	
3.β thalassemia major3	75.3	1.7		68.4	2	
4.Homozygous/ double heterozygous for β-thal assemia and δβ- thalassemia1	93.9	1.1		93.4	1.2	
5.Homozygous/ double heterozygous for β-tha lassemia and δβ- thalassemia2	92.5	2.2		15.9	3	
6.Sickle cell anemia	24.4	2.3	HbS 67.6	26.2	1.6	HbS 71.4
7.Compound heterozygous SCA and β thalassemia	15.6	5.6	HbS 58.4	18.9	4.5	HbS 66.8
8.Compound heterozygous for HbE/β-thalassemia	6.3	7.7		5.2	4.1	HbE 3.9

Apart from β thalassemia trait and normal cases which form bulk of the cases, we got few cases which are different from the usual one about which we have mentioned in table8 along with values of different Hb variants obtained by each modality.

Table9. Table showing comparision of HbF values using HPLC and electrophoresis in 8 unusual cases:

Hemoglobinopathies	HPLC	Electrophoresis
1.β thalassemia major1	91.2	91.1
2.β thalassemia major2	93.1	91.8
3.β thalassemia major3	75.3	68.4
4.Homozygous/ double heterozygous for β-thalassemia and δβ-thalassemia1	93.9	93.4
5.Homozygous/ double heterozygous for β-thalassemia and δβ-thalassemia2	92.5	15.9
6.Sickle cell anemia	24.4	26.2
7.Compound heterozygous SCA and β thalassemia	15.6	18.9
8.Compound heterozygous for HbE/β-thalassemia	6.3	5.2

Fig26. Line diagram showing comparision of HbF values using HPLC and electrophoresis in 8 unusual cases:

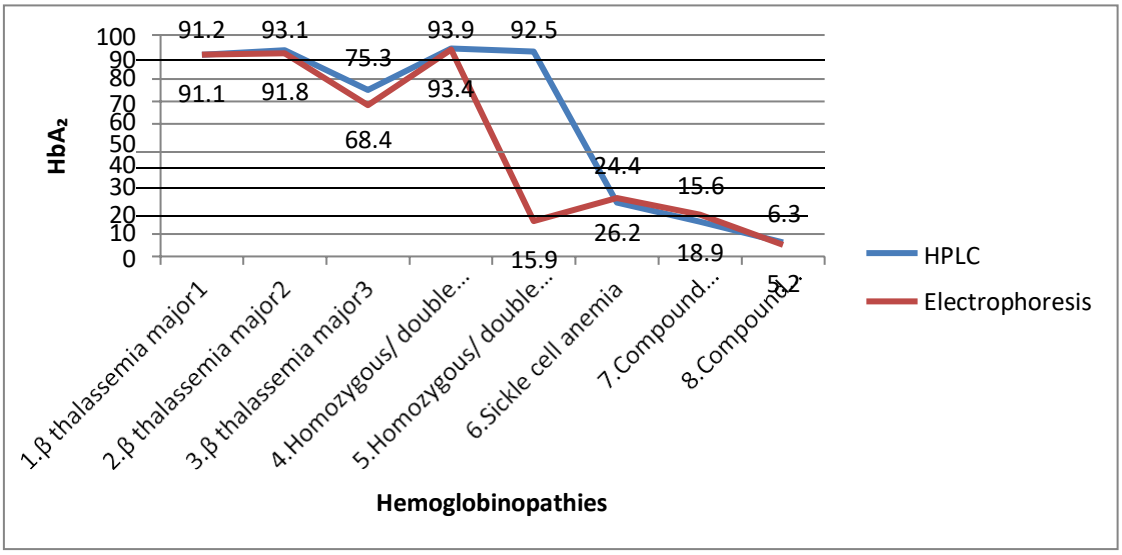
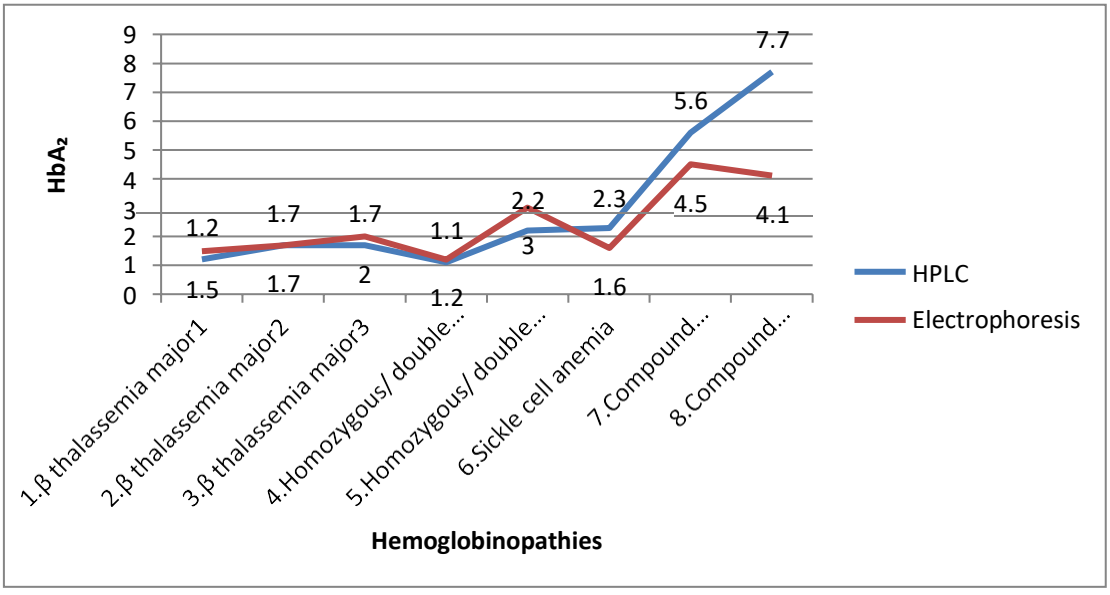


Table10. Table showing comparision of HbA_2 values using HPLC and electrophoresis in 8 unusual cases:

Hemoglobinopathies	HPLC	Electrophoresis
1.β thalassemia major1	1.2	1.5
2.β thalassemia major2	1.7	1.7
3.β thalassemia major3	1.7	2
4.Homozygous/ double heterozygous for β-thalassemia and δβ-thalassemia1	1.1	1.2
5.Homozygous/ double heterozygous for β-thalassemia and δβ-thalassemia2	2.2	3
6.Sickle cell anemia	2.3	1.6
7.Compound heterozygous SCA and β thalassemia	5.6	4.5
8.Compound heterozygous for HbE/β-thalassemia	7.7	4.1

Fig27. Line diagram showing comparision of HbA_2 values using HPLC and electrophoresis in 8 unusual cases:



DISCUSSION

According to WHO around 5% of World's population are carriers for genetic hemoglobin disorder[2]. In India frequency of β-thalassemia in general population is 3.5% to 15%[6]. Whereas prevalence of β-thalassemia carriers in Karnataka is 2.16% [8].

Most common inherited diseases worldwide are Hemoglobinopathies. It is important to know about the prevalence of β-thalassemia in India due to socio-cultural practices, marriages in the same ethnic group/caste as it is an autosomal recessive disorder.β-thalassemia is more common in certain communities such as Sindhi, Gujarati, Bengali and Punjabi in India with an incidence of 1-17%[2].

Study included 41 cases out which most cases(71%) were between the age group of 20-35yrs followed by 17% cases of 2-20yrs and 12% cases of <2yrs.

Gender predilection: In our study patients between age group 20-35yrs, they are parents of known β-thalassemia major which includes mostly mothers accompaning the children hence female predilection is observed.

CBC parameters of pts. with hemoglobinopathies : CBC parameters in our study were well correlated with the CBC parameters of other studies. Mean Hb concentration of β-thalassemia trait is towards lower limit while compared to other hemoglobinopathies which showed significant decrease in Hb concentration.

It appeared that MCV and MCH were almost similar in β-thalassemia major and β-thalassemia trait which is consistent with the study conducted by Rachna Khera et al[3].

The p value in Table2 was calculated by using student's T test, which was significant for both MCV($p<0.001$) and MCH($p<0.001$). Except for β-thalassemia trait and

normal cases all other cases showed increase in observed reticulocyte count (Normal observed reticulocyte count=0.5-2.5%).

Table11. Studies showing increased mean RBC count in β-thalassemia trait:

Study	Mean RBC count
Present study	481561±15522
Rachna et al[3]	518000±27000
Meenakshi M et al[9]	452000±12500
P C Giordano et al[23]	600000±10000

Table 12. Comparision of CBC parameters between present study and study done by Rachna et al[2]:

	Present study			Rachna et al[2]		
	Hb	**MCV**	**MCH**	**Hb**	**MCV**	**MCH**
Beta Thalassemia Trait	10.9±1.8	62.4±4.1	19.3±2.1	9.3±2.7	62.7±7.7	19.56±2.5
Beta Thalassemia Major	5.7±1.4	68.6±1.3	21.6±0.7	5.03±1.9	64.9±7.9	20±1.9
Sickle Cell Anemia	7.5	87.2	31	6.4	64.2	17.4
HbS/ β-thalassemia	9.3	64.5	21.6	8.4±0.4	64.4±5.4	21.8±1.8
HbE/ β-thalassemia	7.4	63.1	20.4	4.9±1.9	71.7±5.8	19.6±1.8

β-thalassemia trait: In our study we diagnosed maximum number of β-thalassemia trait cases(58.5%) followed by normal(22.3%), β-thalassemia major(7.2%), Homozygous/ double heterozygous for β-thalassemia and δβ-thalassemia(4.8%).And also got 1 case each of sickle cell anemia, compound heterozygous sickle cell anemia and β-thalassemia and compound heterozygous for HbE/ β-thalassemia.

Prevalence of different hemoglobinopathies in a certain population was correlating with many other studies few of them are Rachna K et al[3], Santosh Kumar Mondal et al[31] and Prakash Kumar Mandal et al[7].

Table13. Prevalence of different hemoglobinopathies in comparision with other studies:

Study	Present study	Monica Dogaru et al[1]	Rachna et al[3]	Santosh Kumar Mondal[31]
BTT	75%	43.8%	56.3%	63%
BTM	9.375%	1.32%	5.45%	5.5%
HDHBD	6.250%	0.99%		

BTT: β-thalassemia trait, BTM: β-thalassemia major, HDHBD: Homozygous/ double heterozygous for β-thalassemia and δβ- thalassemia

This high incidence of β-thalassemia trait suggests that proper screening should be done for women of reproductive age so as to prevent cases of β-thalassemia major in the next generation.

Sarika Verma et al concluded that conditions wherever β-thalassemia trait is co-existing with iron decificiency anemia there HbA_2 value is usually low, which can be wrongly interpreted as normal either by HPLC or Hb electrophoresis[32]. In our study we got 9 normal cases out of which 2 cases were interpreted as normal with HPLC was normal whereas low HbA_2 with electrophoresis.

Normal cases: Many indices such as Mentzer(1973), England and Fraser(1973), Srivastava and Bevington(1973), Shine-Lal(1977) and Green-King(1989) were used to differentiate between iron deficiency anemia and β-thalassemia trait, but amongst

them Mentzer's index was concluded as the best by M A Ehsani et al[26]. In our study we got 2 such cases in which that dilema was there, so for those 2 cases we used Mentzer's index. In both the cases Mentzer's index came out to be >13 which means iron deficiency anemia whereas HPLC was normal and electrophoresis gave result as low HbA_2. So for those 2 cases serum ferritin was suggested.

β-thalassemia major: Mutation in β-globin gene on Ch.11 causes β-thalassemia major[2]. β-thalassemia major present with clinical symptoms <2yrs of age and are transfusion dependent[2], in our study 3 cases of β-thalassemia major are detected, all are <2yrs of age.

In these 3 cases of β-thalassemia major HPLC didn't gave exact value of HbF because the HPLC model which we are using in our laboratory i.e. Bio-Rad D10(extended program); basic range of HbF which it can calculate is 0.8-16.5% beyond which it goes and elutes with $LHbA_1c$; so the values are mentioned as HbF in the table8 are of$LHbA_1c$ whereas CZE gives exact value of HbF which is very helpful to differentiate between β-thalassemia major and intermedia.

Sickle cell anemia: We had 2 cases of hemoglobinopathies with HbS component were HbA_2 values were higher in HPLC as compared to CZE. In a study David F Kerenet al[21] concluded that in hemoglobinopathies having HbS component the value of HbA_2 was higher in HPLC as compared to CZE because of comigration of glycated products of HbS with HbA_2[21]. HbS values in such cases were higher with Capillary zone electrophoresis as compared to HPLC[21]. Silvana Fahel da Fonseca et al suggests to use a secondary confirmatory test complementary to HPLC for diagnosing HbS/ β^o thalassemia.

Harteveld et al described sickle test which will produce false negative results when executed badly but will never give false positive results. It is a confirmatory test in patients and carriers including newborns when performed properly[29].

Compound heterozygous for HbE/β- thalassemia: Chatterjee et al reported first case of HbE disease in India[33]. According to Olivieri NF et al Indian subcontinent is having many cases of HbE trait and compound heterozygous for HbE/β- thalassemia[34]. A multicenter study conducted in Dibrugarh(Assam) incidence of HbE trait was between 41.1-66.7%[19].β chain mutation(β26 Glu→Lys) causes HbE thalassemia[2].

HbE co-elutes with HbA_2 in HPLC making it difficult to segregate and quantify HbA_2 in such samples whereas it is not so with CZE; CZE has the potency to separate HbE from HbA_2 which is seen in the case of β-thalassemia/ HbE which we got in our study, this finding is also consistent with the study conducted by David F Keren et al[21]. Our study in comparision with the study conducted by Watchareeet al[17] gave slightly different results in evaluating HbA_2 values in cases of β-thalassemia/ HbE with CZE. In that study they got HbA_2>6% while in our study we got it as <6%. Whereas there was no significant difference in evaluating HbF by both the modalities i.e. HPLC and CZE in their study as well as in our study in evaluating cases of Compound heterozygous for HbE/ β-thalassemia.

In all those cases which had Hb variants such as HbS, HbE, etc, HbA_2 value was more in HPLC as compared to CZE. Studies favouring this finding are Silvana Fahel da Fonseca et al[30] and Greene et al[35].

For all those infants in which interpretation was difficult repeat HPLC and electrophoresis after 1yr of age along with parental screening was suggested. In such conditions parental screening is preferred over molecular analysis.

Abnormal Hb variants(HbS,HbE,etc) should be analysed by electrophoresis (modality other than HPLC) for confirmation, according to recent guidelines[5] and is also consistent with the study conducted by David F Keren et al[21].

SUMMARY

A prospective hospital based study was conducted in the department of Pathology(Central laboratory), BLDEU'S Shri B.M.Patil Medical College, Hospital and Research centre, Vijayapura; during: 1st December 2018 to 30th May 2020.

A total of 41 adults and children blood samples were examined for routine thalassemia screening using both the modalities i.e. HPLC and CZE. All samples were analysed using automated cell counter (Sysmex XN-1000), HPLC (Biorad D-10) and capillary zone electrophoresis.

Out of 41 cases, most common hemoglobinopathy observed in our study was thalassemia trait(24) followed by 3 cases of β-thalassemia major, 2 cases of homozygous/ double heterozygous for β-thalassemia and δβ-thalassemia and 1 case each of sickle cell anemia, Compound heterozygous sickle cell anemia and β-thalassemia and compound heterozygous for HbE/ β-thalassemia. In our study we got 9 normal cases.

HbF 10%-30% indicates it is a case of thalassemia intermedia whereas HbF 30%-90 %indicates thalassemia major.

When HbF value is >16.5% it merges with HbA1c values in HPLC(Bio-Rad D-10) because of which it is very difficult to comment about exact values of HbF with the help of HPLC. So for such cases CZE helped us to evaluate exact value of HbF.

Analysis of 41cases in which 33 cases showed correlation of HbF, HbA and HbA_2 values with both HPLC and CZE. While in 8 cases CZE gave exact quantification of Hb variants which helped to come to final diagnosis.

CONCLUSION

- Exact diagnosis of Hb variants is essential for proper management.
- HPLC and CZE helps in quantification of Hb variants.
- Qunatification of Hb variants was better with CZE except for beta thalassemia trait and normal cases in which both the modalities gave same results.
- Advantage of Hb electrophoresis over HPLC is that it gives exact values of HbF which is very essential to differentiate thalassemia intermedia from thalassemia major.
- CZE exactly evaluates percentage of HbE which elutes with HbA_2 in case Compound heterozygous for HbE/β-thalassemia in HPLC
- CZE is complimentary to HPLC, which is very helpful for evaluating exact values of HbF, HbS and HbE.
- Cases with low HbA_2 should be subjected to iron status study to rule out co-existing IDA.

Limitations:

- HPLC is very costly, needs proper skill, experience and knowledge for interpreting the results.
- It is difficult to make out normal HbA_2, α-thalassemia and rare hemoglobinopathies with HPLC because they elute with similar retention time.
- Sample size is less.
- Interpretation of HPLC is affected by glycated Hb and IDA.

Recommendations:

- Prenatal screening should be made mandatory in high prevalent areas of hemoglobinopathies to reduce no.of cases of β-thalassemia major.
- Genetic sequencing is recommended in few cases to confirm the Hb variants.

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ANNEXURE-I

ETHICAL CLEARANCE

B.L.D.E (Deemed to be University)
SHRI.B.M.PATIL MEDICAL COLLEGE HOSPITAL & RESEARCH CENTRE
VIJAYAPUR – 586103

IEC/NO:286/2018
17-11-2018

INSTITUTIONAL ETHICAL COMMITTEE

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this college met on 13-11-2018 at 03-15 PM scrutinize the Synopsis of Postgraduate Students of this college from Ethical Clearance point of view. After scrutiny the following original/corrected and revised version synopsis of the Thesis has accorded Ethical Clearance.

Title : Comparative study between HPLC and Hb electrophoresis to characterize hemoglobin profile in hemoglobinopathies.

Name of P.G. Student : Dr K.Pallavi.
Department of Pathology.

Name of Guide/Co-investigator: Dr. R.M.Potekar, Professor of Pathology.

DR.RAGHAVENDRA KULKARNI
CHAIRMAN
Institutional Ethical Committee
BLDEU's Shri B.M. Patil
Medical College,VIJAPUR-586103.

Following documents were placed before E.C. for Scrutinization:

1) Copy of Synopsis/Research Project
2) Copy of informed consent form.
3) Any other relevant documents.

ANNEXURE-II

B.L.D.E (DEEMED TO BE) UNIVERSITY, SHRI B.M.PATIL MEDICAL COLLEGE HOSPITAL AND RESEARCH CENTER, VIJAYAPURAA-586103

INFORMED CONSENT FOR PARTICIPATION IN DISSERTATION/RESEARCH

I, the undersigned,_____, S/O D/O W/O_____, aged_____years, ordinarily resident of_____do hereby state/declare that Dr _____ of Hospital has examined me thoroughly on_____at _____(place) and it has been explained to me in my own language that I am suffering from_____disease (condition) and this disease/condition mimic following diseases. Further Doctor informed me that he/she is conducting dissertation/research titled_____under the guidance of Dr _____ requesting my participation in the study. Apart from routine treatment procedure, the pre-operative, operative, post-operative and follow-up observations will be utilized for the study as reference data.

Doctor has also informed me that during conduct of this procedure adverse results may be encountered. Among the above complications most of them are treatable but are not anticipated hence there is chance of aggravation of my condition and in rare circumstances it may prove fatal in spite of anticipated diagnosis and best treatment made available. Further Doctor has informed me that my participation in this study will help in evaluation of the results of the study which is useful reference to treatment of other similar cases in near future, and also I may be benefited in getting relieved of suffering or cure of the disease I am suffering.

The Doctor has also informed me that information given by me, observations made/ photographs/ video graphs taken upon me by the investigator will be kept secret and not assessed by the person other than me or my legal hirer except for academic purposes.

The Doctor did inform me that though my participation is purely voluntary, based on information given by me, I can ask any clarification during the course of treatment / study related to diagnosis, procedure of treatment, result of treatment or prognosis. At the same time I have been informed that I can withdraw from my participation in this study at any time if I want or the investigator can terminate me from the study at any time from the study but not the procedure of treatment and follow-up unless I request to be discharged.

After understanding the nature of dissertation or research, diagnosis made, mode of treatment, I the undersigned Shri/Smt _____ under my full conscious state of mind agree to participate in the said research/dissertation.

Signature of patient:

Signature of doctor:

Witness: 1.

2.

Date:

Place:

ANNEXURE-III

PROFORMA

Name	:	OP/IP No.	:
Age	:		
Sex	:	D.O.A	:
		D.O.D	:
Residence	:		

Presenting Complaints :

No. of blood transfusions till date :

1st blood transfusion at age of :

Similar history in family :

General physical examination:

Systemic examination:

Clinical Diagnosis:

INVESTIGATIONS:

Haematological investigations:

CBC (Complete Blood Count):

Parameters	
RBC count	
Hb	
HCT	
MCV	
MCH	
MCHC	
ESR	
RDW	
nRBC	

Reticulocyte count:

a.Reticulocyte count:

b.Corrected reticulocyte count:

c.Reticulocyte production index:

Peripheral Smear Examination:

Hemoglobin variant analysis by HPLC:

HbF

HbA

HbA1C

Others

HPLC Result:

Hb electrophoresis:

HbF

HbA

HbA1C

Others

Haemolytic profile:

Sickling test:

NESTROF test:

Diagnosis:

KEY TO MASTER CHART

BEH	:	Borderline elevated HbA2 level to the age of the pt.
BTI	:	Beta thalassemia intermedia
BTM	:	Beta thalassemia major
BTT	:	Beta thalassemia trait
CHEB	:	Compound Heterozygous for HbE/β-thalassemia
CHSB	:	Compound Heterozygous SCA and β-thalassemia
CRC	:	Corrected Reticulocyte Count
F	:	Female
Hb	:	Hemoglobin
HBT	:	Homozygous β-thalassemia
HCT	:	Hematocrit
HDHBD	:	Homozygous/ Double Heterozygous for Beta thalassemia and Delta beta thalassemia
HPLC	:	High Pressure Liquid Chromatography
HSD	:	Homozygous HbS(Sickle cell) disease
LHL	:	low HbA2 level
M	:	Male
MCH	:	Mean Corpuscular Hemoglobin
MCHC	:	Mean Corpuscular Hemoglobin Concentration
MCHC	:	Microcytic Hypochromic anemia
MCV	:	Mean Corpuscular Volume
Mon.	:	Months
NCNC a.	:	Normocytic Normochromic
NCNC	:	Normocytic Normochromic smear

ND	:	Not Determined
nRBC	:	Nucleated Red Blood Cell
NS	:	Normal study
OPD no.	:	Out patient department
PS	:	Peripheral Smear
Pt.	:	Patient
RBC count	:	Red Blood Cell count
RC	:	Reticulocyte count
RDW	:	Red cell Distribution Width
RPI	:	Reticulocyte Production Index
SCA	:	Sickle cell anemia
Yr	:	Year

MASTER CHART

Name	Age	Sex	Lab No.	RBC count	Hb	HCT	MCV	MCH	MCHC	RDW	nRBC	RC	CRC	RPI	HbF	HbA0	HbA2	HbA1C	Others	HPLC	HbF	HbA	HbA2	Others	Hemoglobin Electrophoresis	Advise	PS
Renuka Madar	20	F	50828/19	5,78,000	11.5	38.3	66.3	19.9	30	15.6	0	0.92	0.88	0.59	2.9	80.4	4.7	5.5		BTT	2.2	92.8	5		BTT	Family studies	MCHC
Sitabai Yallappa	26	F	230453/18	5,42,000	10.6	34.3	63.3	19.6	30.9	16	0	1.29	1.1	0.55	0.8	81.1	5.6	5.6		BTT	ND	94.8	5.2		BTT	Family studies	MCHC
Annapurna Goundi	22	F	230454/18	4,52,000	8.7	28.5	63.1	19.2	30.5	15.6	0.2	1.97	1.4	0.7	1.1	82.3	5.2	5		BTT	0.5	94.3	5.2		BTT		MCHC
Savita Biradar	25	F	230455/18	6,53,000	12.5	41.7	63.9	19.1	30	18.6	0	0.61	0.63	0.63		82.2	4.8	5.8		BTT		95.4	4.6		BTT	Family studies	MCHC
Gangu Kulekumatagi	30	F	230457/18	4,60,000	6.8	24.4	53	14.8	27.9	20.4	0	0.59	0.36	0.18	<0.8	82.7	4.4	5.4		BTT		95.6	4.4		BTT		MCHC
Vidya Pujari	24	F	13273/19	5,42,000	9.9	32.9	60.7	18.3	30.1	16.3	0	0.82	0.67	0.33	<0.8	82.3	5.3	5.7		BTT		94.7	5.3		BTT	Family studies	MCHC
Siddaram	32	M	230447/18	6,67,000	13.5	43	64.5	20.2	31.4	18.2	0	1.59	1.7	1.7	<0.8	80.9	7	5.3		BTT		93.4	6.6		BTT	Family studies	MCHC
Nirmala	24	F	13272/19	4,98,000	8.9	29.4	59	17.9	30.3	19.8	0	1.42	1.04	0.57	<0.8	81.4	6.8	5.3		BTT		93.1	6.9		BTT	Family studies	MCHC
Satya Singe	25	F	230440/18	5,24,000	10.1	32.1	61.3	19.3	31.5	16.8	0	1.62	1.3	0.65	<0.8	83.1	5.5	4.7		BTT		95	5		BTT	Family studies	MCHC
Gayatri Rathod	23	F	230441/18	5,77,000	10.6	35	60.7	18.4	30.3	18.8	0	0.78	0.68	0.45	0.9	79.5	3.5	6.2		BTT		95.8	4.2		BTT		MCHC
Pujappa Hachadad	32	M	230439/18	5,36,000	10.8	34.2	63.8	20.1	31.6	18.9	0	1.27	1.09	0.54	<0.8	81.3	6	5.4		BTT		94	6		BTT	Family studies	MCHC
Gangappa Medegar	31	M	230438/18	6,65,000	12.8	41	61.7	19.2	31.2	18.4	0	1.32	1.35	1.35		81.2	5.2	6		BTT		94.8	5.2		BTT	Family studies	MCHC
Sharada Chavan	35	F	230437/18	5,77,000	10.9	35.9	62.2	18.9	30.4	18.1	0	1.28	1.15	0.77	<0.8	80.2	4.6	6.5		BTT		95.4	4.6		BTT	Family studies	MCHC
Roopa Naik	24	F	230436/18	5,70,000	10.9	36.1	63.3	19.1	30.2	16	0	0.64	0.58	0.39	<0.8	82.8	5.3	5.1		BTT		94.8	5.2		BTT	Parental studies	MCHC

Monika	24	F	12533/19	5,16,000	10	32.8	63.6	19.4	30.5	16.3	0	0.81	0.664	0.33	1.1	82.3	5.3	5.3		BTT	0.3	94.6	5.1		BTT	Family studies	MCHC
Ashwini	7mon.	F	230561/18	2,90,000	7.9	23.1	72	27.2	34.2	14.1	3.2	1	0.68	0.27		81.7	3.7	6.5		BTT		97.3	2.7		BTT	Clinical correlation	MCHC
Venkatesh K	31	M	78673/18	6,66,000	13.4	45.1	68.3	20.3	29.7	18.9	0	1.17	1.31	1.31	<0.8	82.1	5.5	5.9		BTT		94	6		BTT	Family studies	MCHC
Geeta K	25	F	78675/18	6,03,000	11.3	38.3	64.3	18.7	29.1	16.7	0	0.84	0.81	0.54	<0.8	82.3	5.3	5.3		BTT		95	5		BTT	Family studies	MCHC
Nanteshri	24	F	12532/19	5,81,000	10.4	34.4	59.2	17.9	30.2	17.8	0	1.87	1.6	0.8	1.6	83	5	4.7		BTT	1.1	93.9	5		BTT	Family studies	MCHC
Santosh	33	M	12536/19	6,76,000	13.7	44.5	65.8	20.3	30.8	19.4	0	1.69	1.88	1.88	1.3	81.5	5.8	5		BTT	0.4	93.9	5.7		BTT	Family studies	MCHC
Davalpur	33	M	13271/19	6,92,000	14.1	46.8	67.6	20.4	30.1	18	0	0.95	1.11	1.11	<0.8	82.6	4.7	5.7		BTT		95.5	4.5		BTT	Family studies	MCHC
Jannat	26	F	69865/19	5,79,000	10.7	35.5	61.3	18.5	30.1	17	0	1.54	1.36	0.91	<0.8	82.5	4.8	5.2		BTT		94.8	5.2		BTT	Family studies	MCHC
Suresh	5	M	69864/19	6,33,000	10.5	34.3	54.2	16.6	30.6	19.7	0	0.88	0.81	0.42	2.1	78.4	5.4	5.7		BTT		93.5	6.5		BTT	Family studies	MCHC
Sanju Madar	25	M	50829/19	6,20,000	11.6	37.9	61.1	18.7	30.6	16.3	0	0.91	0.86	0.57	0.8	82.7	4.7	5.6		BTT		94.9	5.1		BTT	Family studies	MCHC
Madivallava P	26	F	230450/18	4,23,000	8.9	30.3	71.6	21	29.4	17.8	0	0.69	0.52	0.26		83.8	2.2	5.7		NS		98.1	1.9		LHL	Serum iron studies	MCHC
Shabana	30	F	12534/19	5,25,000	12.1	39.8	75.8	23	30.4	15.5	0	0.97	0.96	0.64	<0.8	84.7	2.2	5.9		NS		98.3	1.7		LHL	Serum iron studies	MCHC
Haseena B	30	F	12530/19	6,13,000	17.3	53.5	87.3	28.2	32.3	12.6	0	0.55	0.73	0.73	<0.8	85.1	2.9	5.4		NS		97.3	2.7		NS		NCNC
Nayan	30	F	100231/18	4,97,000	11.7	36.1	72.6	23.5	32.4	15	0	1.4	1.26	0.84	<0.8	83.7	2.4	5.7		NS		97.8	2.2		NS		NCNC
Basalingamma M	24	F	75024/18	4,30,000	11.9	39.5	91.9	27.7	30.1	13.4	0	0.8	0.79	0.395	<0.8	96.1	3.1	4.7		NS		98	2		NS		NCNC
Manappa H	30	M	75023/18	5,04,000	14.3	41.3	81.9	28.4	34.6	12.7	0	1.38	1.425	1.425	<0.8	96.4	2.8	5		NS		98.2	1.8		NS		NCNC
Vinaykumar	7	M	241118/18	3,93,000	6.2	24.3	61.8	15.8	25.5	25.2	0	3.11	1.88	0.75	<0.8	85.9	2.3	4.5		NS		97.5	2.5		NS	Parental studies	MCHC
Padma Guddodagi	24	F	230444/18	3,62,000	11.4	35.2	97.2	31.5	32.4	13.2	0	1.16	1.02	0.68		84.6	3.3	5.2		NS		97	3		NS		NCNC

Veeresh Ramaling	6mon.	M	227860/19	1,24,000	3.6	9	72.6	29	40	29.4	109.4	4.16	1.069	0.356	13.4		3.3	8.6		NS	13.9	82.4	3.7		NS	Clinical correlation	MCHC
Virat Ramesh	4	M	75565/18	3,02,000	6.6	20.3	67.2	21.9	32.5	33.2	1.64	3.31	1.81	0.72	73.5	5.8	1.2		HbA1b-17.7	BTM	91.1	7.4	1.5		BTM	1.Clinical correlation	MCHC
																										2.Parental screening	
Virat	12	M	99321/19	2,89,000	6.4	20	69.2	22.1	32	32.7	5.8	5	2.7	1.08	74.7	4.9	1.7		HbA1b-18.4	BTI	91.8	6.5	1.7		BTM	1.Clinical correlation	MCHC
																										2.Parental screening	
Shivani Sanju Madar	10 mon.	F	48784/19	1,97,000	4.1	13.7	69.5	20.8	29.9	36.5	1.83	4.72	1.61	0.53	62.4	18.7	1.7		HbA1b-12.9	HDHBD	68.4	29.6	2		BTM	1.Clinical correlation	MCHC
																										2.Parental studies	
Mariyam	3 mon.	F	208541/18	1,83,000	4.5	15	82	24.6	30	34.9	13.9	7.75	3.3	1.32	93.9	3.4	1.1	0	P3-1.6	HDHBD	93.4	5.4	1.2		HBT	Parental studies	NCNCa.
Kiran Chandrashekar	6mon.	M	163188/19	1,69,000	3.2	9.6	56.8	18.9	33.3	36.7	0.64	1.43	0.39	0.13	92.5	3.7	2.2			HDHBD	15.9	81.1	3		BEH	1.Parental screening	MCHC
																										2.DNA analysis	
Praveen P Rathod	12	M	2304/20	2,42,000	7.5	21.1	87.2	31	35.5	16.2	2.7				24.4	3.7	2.3		HbS-67.6	SCA	26.2	0.8	1.6	HbS-71.4	SCA	1.Parental screening	NCNC
Santosh Daku Rathod	28	M	232629/18	4,31,000	9.3	27.8	64.5	21.6	33.5	20.6	27.3	4.06	2.8	1.4	15.6	18.6	5.6	6.2	HbS-58.4	CHSB	18.9	9.8	4.5	HbS-66.8	1.HSD	Family studies	MCHC
																									2.CHSB		
Akibh Jatagar	2	M	69984/20	3,63,000	7.4	22.9	63.1	20.4	32.3	23	2.8	2.93	1.81	0.724	6.3	3.7	7.7	5.2	HbA0-73	BTT	5.2	86.8	4.1	HbE-3.9	CHEB	1.Parental screening	MCHC
																										2.Clinical correlation	