

HbA₂ and Fetal haemoglobin in The Diagnosis of Thalassemia and Hemoglobinopathies

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ABSTRACT

Background: Hemoglobin (Hb) disorders which include hemoglobinopathies and Thalassemia affect 7% of the world population. Capillary electrophoresis is useful for screening and follow up of Hb disorders.

Aim: To evaluate HbA₂ and HbF (fetal hemoglobin) in the diagnosis of Thalassemia and hemoglobinopathies.

Material and Methods: 100 consecutive Capillary Hemoglobin electrophoresis done as a part of screening programme for Hb disorders from Jan 2016 to June 2016 was included in the study. Children <1 yr of age and individuals with recent blood transfusion were excluded. Hb, RBC count and MCV were recorded.

Results: Of the 100 Hb electrophoresis performed, 57 normal and 43 abnormal patterns were seen. Among the abnormal Hb patterns, β thalassemia trait (β T) was the most common constituting 58.2% followed by Sickle cell (HbS) trait (11.7%), HbE trait (9.3%) and HbE/ β Thalassemia (7.0%). The HbA₂ levels in normal, β T, Sickle cell trait, HbE trait and HbE/ β thalassemia were 2.12% (SD 0.5), 4.9% (SD 0.62), 3.28% (SD 0.43), 3.98% (SD 0.67) respectively. The difference in HbA₂ were significant (p value <0.0001). The difference in HbA₂ levels between HbE trait and HbE disease was also significant (p value 0.036). Based on the HbF levels Sickle cell hemoglobinopathy was further classified.

Conclusion: The difference in HbA₂ levels in normal subjects, Thalassemia and hemoglobinopathies are statistically significant. The percentage of HbF in sickle cell gives information about coexisting hemoglobin disorder. In HbE hemoglobinopathy, HbA₂ along with HbF identifies a specific group HbE/ β Thalassemia which often needs clinical intervention.

Keywords: Hemoglobin A₂, Fetal Haemoglobin, Hemoglobinopathy, Thalassemia, Capillary Electrophoresis

Introduction

Hemoglobin (Hb) disorders are a frequent genetic disease affecting 7% of world population.^[1] Hemoglobinopathies result from a structural defect in the globin chain where as Thalassemias are due to a quantitative defect in the globin chain production. They are detected in populations during programmes run for prevention of Hb disorders or in patients with clinical suspicion or familial history of Hb disorder. Capillary electrophoresis has been used for precise and accurate first line screening and follow up Hb disorders.^[2] DNA or protein analysis are recommended for a definitive diagnosis in difficult cases. HbA₂ and HbF along with the specific abnormal band, point to the diagnosis of Hb disorder. Few studies have separately evaluated HbA₂ and HbF in Hb disorders.^[3,4] Capillary electrophoresis (CE) and high performance liquid chromatography (HPLC) were used respectively for hemoglobin separation respectively in these studies. But both HbA₂ and HbF have not been evaluated together in Hb disorders by CE. So the present study was undertaken

to evaluate HbA₂ and HbF in the diagnosis of Thalassemia and hemoglobinopathies.

Material and Methods

100 consecutive cases of Hb electrophoresis done as a part of screening programme for hemoglobin disorders from Jan 2016 to June 2016 were included in the study. Children less than one year of age and individuals with a history of blood transfusion in the past three months were excluded in the study. Hemoglobin electrophoresis was done by Sebia Minicap Flex piercing capillary electrophoresis method.^[2] K-EDTA anticoagulated blood was used. Hb, RBC count and MCV were recorded in each case. Reference range by Sebia CE-HbA 96.8% to 97.8% and HbA₂- 2.2% to 3.2%.

Results

Of the 100 Hb electrophoresis performed 57 showed normal pattern (Fig 1) with HbA ranging between 97.1% and 99% (Mean 97.85, SD 0.52) and HbA₂ between 1% and 3.6% (mean 2.12, SD 0.5). HbF of 0.5% was seen in one of the cases. Hb, RBC count and MCV ranged

between 5gm% to 15.8gm% ,4.15to5.4x10¹² /L and 70-118 femtolitres respectively. The lowest HbA2 observed was 1% which corresponded to Hb of 5 gm% and MCV of 70 fl. Marginally elevated HbA2 of 3.6% was seen in the case with MCV of 118 and Hb of 10gm%.

The 43 abnormal electrophoresis pattern included 26 cases of Thalassemia ,8 cases each of sickle cell hemoglobinopathy and HbE hemoglobinopathy, one case of HbD hemoglobinopathy. Further classification and distribution of cases are shown in table 1.

Thalassemia out numbered hemoglobinopathies. The most common abnormal Hb pattern was β thalassemia trait (β TT) (Fig2A). An elevated HbA2 level between 3.8% and 6.2% (4.9, SD 0.62) was observed. HbF was seen in 13 of the 25 cases and varied between 0.3%to 6%.The mean RBC count and MCV were 5.59 x10¹²(SD 0.84) and 64.2 (SD 4.08) (Table 2). A single case of α Thalassemia was seen with HbH band(28.3%) (Fig2B) .HbA of 69.6% , HbA2 of 2.1% were recorded. The Hb, RBC and MCV were 13gm/dl, 4.8x10¹² and 83 fl respectively.

The details of eight cases of Sickle cell hemoglobinopathy are shown in table 3. The five cases of SCT showed HbS band (Fig3A) in addition to HbA. Average HbS was 30.46 (SD7.2) and HbA2 was 3.28 (SD 0.43). HbF was seen in two cases amounting to 0.6% and 1.8%. The mean RBC count and MCV were 4.7x10¹² (SD 0.51) and 81.88

fl (SD 1.1). One case each of Sickle cell disease(SCD) (Fig3B) , heterozygous HbS/ β thalassemia(Fig 4A) and heterozygous HbS/ Hereditary persistence of fetal hemoglobin (HPFH) (Fig 4B) were seen . HbF of 20.6%, 9.0% and 32.8% were seen in these cases respectively. In addition heterozygous HbS/HPFH showed elevated HbA2 levels of 8.4%.

HbE hemoglobinopathy included eight cases (table 4). HbE trait was the most common .Characteristic HbE band was seen (Fig5A) in addition to HbA and HbA2. HbA, HbE and HbA2 were 79.95% (SD 19.6) , 22.9(SD 2.8) and 3.98 (SD 0.67). HbF of 0.6% was seen in one of the cases . The mean RBC count and MCV were 4.99x10¹²(SD 0.65) and 76.1fl (SD 1.33).A single case of HbE disease was present (Fig5B).Three cases showed higher HbF of 11%(SD 1.05) and HbA2 of 4.33(SD 0.15) along with HbE levels of 84.66 (SD 0.97). HbA was nil. They constituted the double heterozygous HbE/ β^0 thalassemia (Fig 5C)

HbD hemoglobinopathy was comprised of a single case of HbD trait as shown in Fig6 with HbD of30.8%, HbA of 66.1% and HbA2 of 3.1% . The Hb, RBC and MCV were 10gm/dl, 4.99x10¹² and 66.7fl respectively. MCV in the ascending order in HbS/ β thalassemia, HbE/ β thalassemia, HbE disease, β TT, HbD, SCD, HbE trait, SCT and α Thalassemia were 52.3fl,62.5fl (SD 1.5),63.4 fl, 64.2 fl (SD 4.08), 66.7fl , 70fl , 76.1fl (SD 1.33), 81.8 fl (SD 1.1) and 83fl respectively.

Table 1: Shows the distribution of Abnormal hemoglobins

Abnormal hemoglobin electrophoresis patterns	No. of cases (n=43)	Percentage
Thalassemia :		
α Thalassemia (HbH)	1	2.3
β Thalassemia trait	25	58.2
Sickle cell hemoglobinopathy :		
Sickle cell disease	1	2.3
Sickle cell trait	5	11.7
Double heterozygous Sickle cell/ β thalassemia	1	2.3
Compound heterozygous HbS/HPFH	1	2.3
HbE hemoglobinopathy	1	2.3
HbE disease	4	9.3
HbE trait	3	7.0
HbE/ β Thalassemia		
HbD hemoglobinopathy	1	2.3

Table 2: Shows electrophoresis pattern, Hemoglobin, RBC count and MCV in Beta Thalassemia Trait.

HbA%	HbA2%	HbF%	Hb(gm/dl)	RBC(x10 ¹² /L)	MCV(fl)
94.3	5.7	---	10.4	5.2	64.3
94.6	5.4	----	11.9	6.16	62.5
88.7	4.8	6.5	8.6	3.68	75.5
95.1	4.9	----	12.2	6.68	59.3
3.4	4.7	1.9	10.1	5.22	62.7

HbA%	HbA2%	HbF%	Hb(gm/dl)	RBC(x10 ¹² /L)	MCV(fl)
96.4	3.8	----	8.4	5.3	64.3
95.5	4.5	----	8.7	4.5	60.5
95.1	4.5	0.9	11	6.4	59.5
94.7	5.3	----	9.8	4.83	60.6
94.6	5.1	0.3	12.5	6.5	62.9
96.2	3.8	----	8.9	4.1	69.7
93.1	6.2	0.7	10.3	5.89	58
94.7	4.8	0.5	9.6	5.28	60.3
95.8	3.8	0.5	11.6	4.85	77
92.9	4.5	2.6	13.1	6.18	68.8
95.1	4.9	-----	12.4	6.6	59.2
94.2	5.5	0.3	12.9	6.61	63.6
94.5	5.1	0.4	11.5	5.4	69.6
95	5	----	8.9	4.64	64
94.6	5.4	----	10.4	5.21	66
93.5	5.7	0.8	11	6.3	63
94.8	5.2	----	12.5	6.6	64
94.3	5.2	0.5	9	6	68
94.7	5	-----	10.5	5.8	63
95.5	4	0.5	10.8	5.9	62.2

Table 3 : Shows electrophoresis pattern, Hemoglobin, RBC count and MCV in Sickle cell Hemoglobinopathy :

Sickle cell hemoglobinopathy (8 cases)	HbA%	HbA2%	HbS%	HbF%	Hb(gm/dl)	RBC(x10 ¹² /L)	MCV(fl)
Sickle cell disease(1)	----	2.8	76.6	20.6	8.3	3.81	70
Sickle cell trait (5)	60.6	3.7	35.7	---	11.1	4.3	80.4
	57.7	2.7	39.6	----	10	4.8	82.6
	74.9	3.2	21.3	0.6	11.5	5.5	82
	69.1	3.1	27.8	----	11	4.7	81.2
	66.6	3.7	27.9	1.8	10.5	4.2	83.2
Double heterozygous Sickle cell/ βThalassemia (1)	5.7	8.4	76.9	9.0	5.2	3.19	52.3
Compound heterozygous HbS/ HPFH (1)	---	1.3	65.9	32.8	10.2	3.27	91.2

Table 4: Shows electrophoresis pattern, Hemoglobin, RBC count and MCV in HbE hemoglobinopathy :

HbE hemoglobinopathy (8)	HbA	HbA2	HbE	HbF	HB	RBC	MCV
HbE disease (1)	---	5.2	94.8	---	8.5	4.2	63.4
HbE trait (4)	71.1	4	24.9	---	11.7	4.8	75.4
	72.3	3.6	24.1	---	14.3	5.9	75
	72.7	3.4	23.9	---	12.1	4.36	78
	75.7	4.9	18.8	0.6	11.9	4.9	76
	---	4.5	84.9	10.6	9	4.7	61
HbE/ β Thalassemia (3)	---	4.2	83.6	12.2	9.2	4.8	64
	---	4.3	85.5	10.2	9.7	5.2	62.5

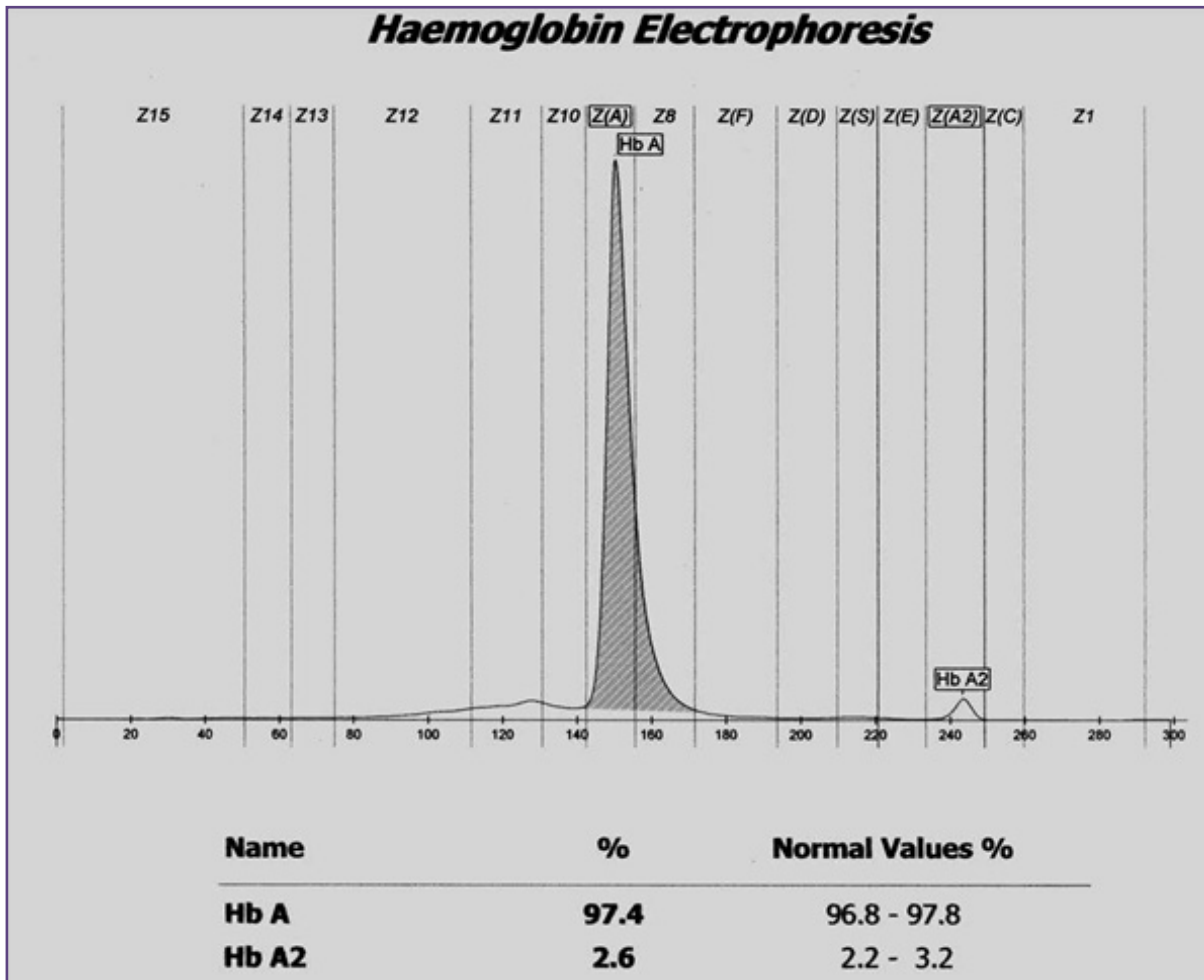


Fig. 1: Shows normal pattern of hemoglobin electrophoresis.

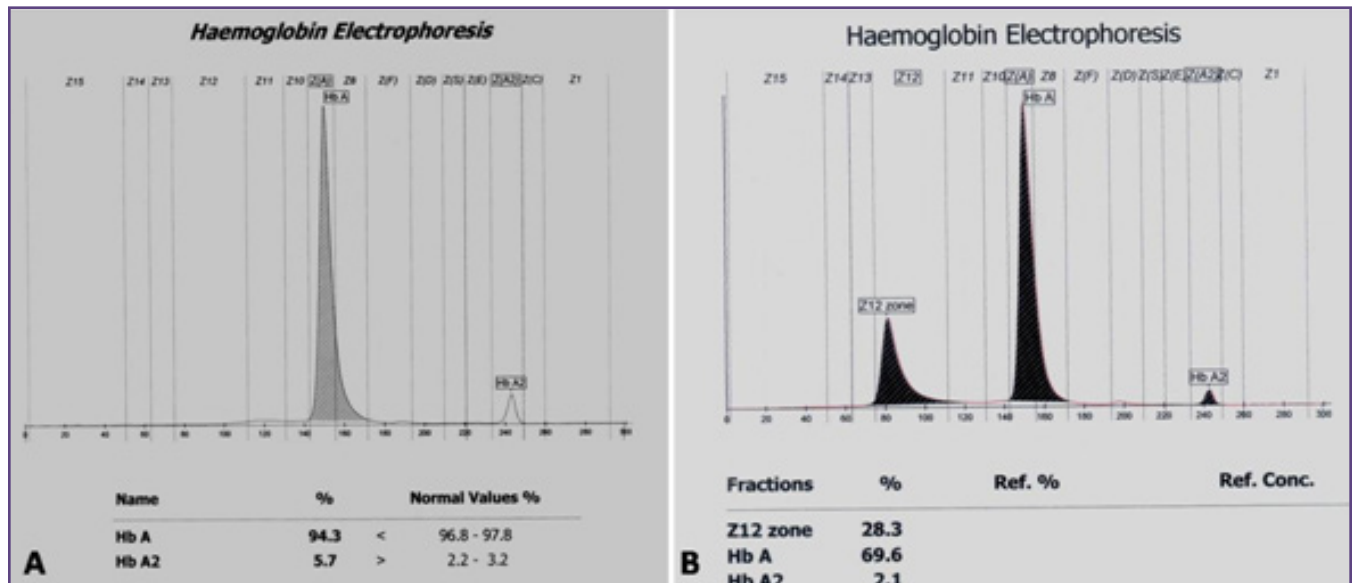


Fig. 2: A Shows elevated HbA2 in beta thalassemia trait. B Shows HbH band in alpha thalassemia.

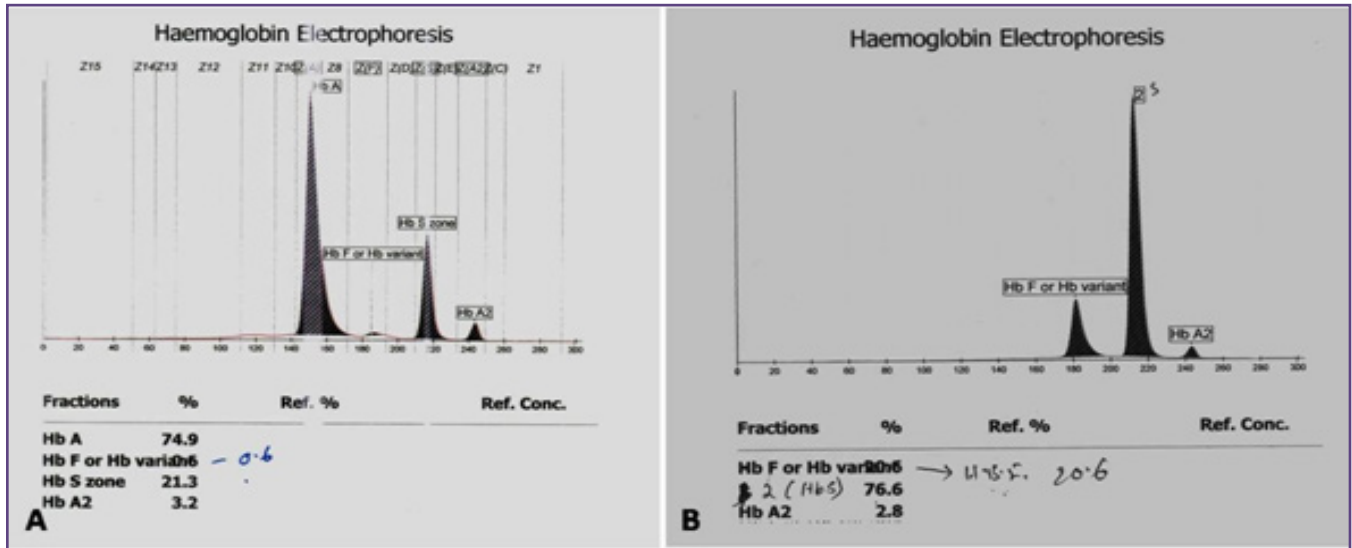


Fig. 3: A Shows HbS in addition to HbA in sickle cell trait. B Shows HbS with HbF in the absence of HbA in Sickle cell disease.

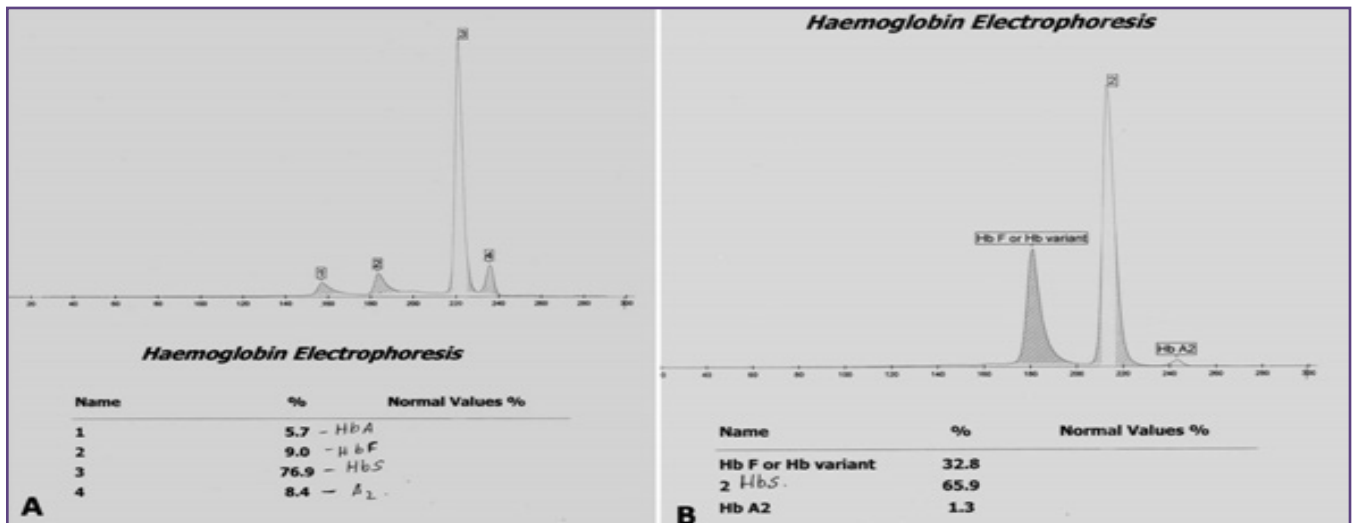


Fig. 4: A Shows elevated HbA2, HbF and HbS in heterozygous HbS- beta thalassemia. B Shows elevated HbF in the presence of HbS in heterozygous HbS-HPFH.

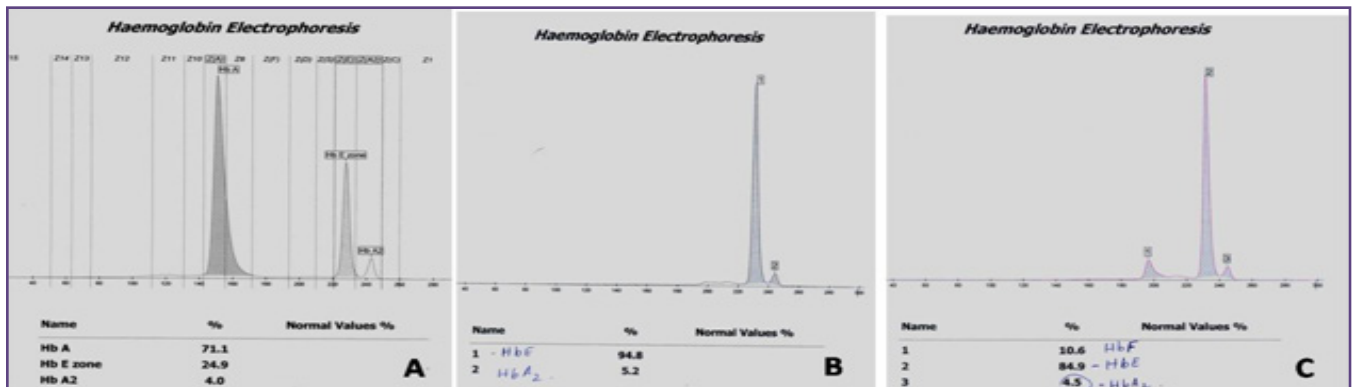


Fig. 5: A Shows HbE in addition to HbA in HbE trait. HbA2 is increased . B Shows HbE only with elevated HbA2 in HbE disease . C Shows elevated HbF and HbA2 in addition to HbE in Heterozygous HbE-beta thalassemia.

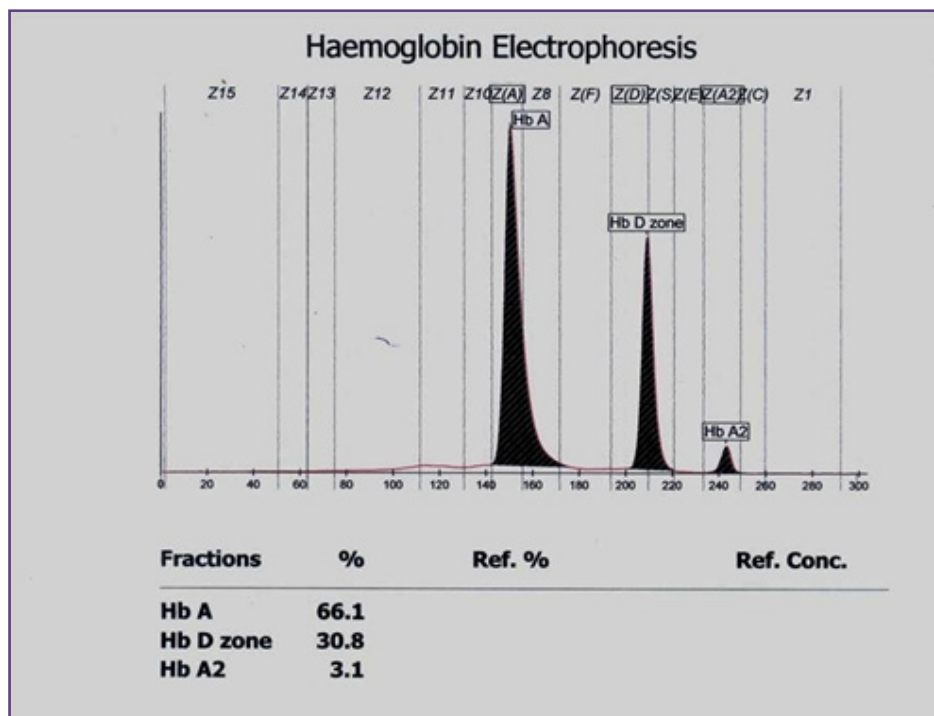


Fig. 6: Shows HbD in addition to HbA and HbA2 in HbD trait.

Discussion

No screening programme is 100% specific and sensitive for the diagnosis of Hb disorders. A combination of haematological, biochemical and molecular analysis including Hb separation on CE, globin chain separation on reverse phase chromatography, sequencing of globin genes, detection of globin gene deletions by PCR and measurement of δ : β globin chain ratios using spectrometry would provide a complete work up for a Hb disorder.^[5] However Assessment of HbA2 and HbF along with specific bands play a vital role in the diagnosis of Hb disorders. CE separates the Hb bands with sufficient clarity compared to the other methods of electrophoresis.^[2,6]

Mean HbA2 levels of 2.12% (SD 0.5) seen in the 57 normal Hb electrophoresis below the mean HbA2 seen in β TT (4.9%) , HbE hemoglobinopathy(HbE trait -3.98%,HbE/ β thal-4.33%) and SCT(3.28%). This difference was statistically significant ($p < 0.0001$). A low HbA2 of 1% was seen in a case of Iron deficiency anemia in the present study . Mosca and others have reported similar findings and have attributed the decreased HbA2 levels in Iron deficiency to the inhibition of δ globin synthesis by low iron levels and to the preferential binding of β to α chain rather than δ chains. One of the cases with MCV of 118fl showed HbA2 level of 3.6% and was a case of VitB12 deficiency. Megaloblastic anemia and hyperthyroidism elevate HbA2 levels .^[7] A

0.5% HbF seen in one of our cases are in accordance with normal adult HbF levels of $< 1\%$.^[4]

β TT was the most common abnormal Hb followed by HbS trait, HbE trait and HbE/ β thalassemia. This is comparable with other studies.^[8,9,10,11] HbS trait was the second most common Hb disorder in the present study while HbE trait occupied the second position in other studies across the Indian population.^[9,10] However Balgir and others have found Sickle cell anemia more common in Orissa, Eastern India.

HbA2 levels in β TT was 4.9% (SD 0.62) (range 3.8%to 6.2%) . A range of 3.5% to 5.5% and 5.23%(SD 0.63) have been seen in other studies using capillary electrophoresis.^[3,5] HbA2 levels upto 6.9% have been observed.^[12] High HbA2 levels over 6.5% characterise a subgroup of β thalassemia caused by deletions that remove the regulatory elements in the promoter region of β gene. These are often accompanied by increase in HbF.^[5] An increase in HbF $> 1\%$ in a healthy adult points to a genetic or acquired pathology.^[4] HbF of 2% and 6.5% were seen in the present study in two cases of β TT . Polymorphisms of BCL11A gene has been associated with high levels of HbF in normal persons , β thalassemia and in sickle cell anemia.^[4,13] Pernicious anemia, aplastic anemia, chronic renal failure and Diabetes mellitus are some of the acquired causes of elevated HbF.^[4]

HbA2 levels in sickle cell trait was(3.28 % ,SD 0.43) significantly more than normal (2.12, SD 0.5) ($p < 0.0001$)

Mean HbA2 levels 0.2% to 1% higher than normal were noted in sickle cell trait by Craver R and others.^[12] The modest increase in HbA2 in SCT is due to the increased avidity of normal δ chains for α chains compared to HbS β chain.^[14] No significant difference in HbA2 between normal subjects and SCT was recorded by Ajjack and others in their study of sickle cell hemoglobinopathy by CE who found a HbS of 39.3% (SD 13.8) in SCT.^[15] This is comparable to HbS of 30.46% (SD 14.4) seen in the present study. Studies show mean HbF levels of 1.4% and 2.14% in SCT.^[13,15] In the present study, fetal hemoglobin was identified in two of the five cases (mean 1.2%)

Fetal Hb is the major modulator of hematological and clinical features in sickle cell disease. HbF levels decline at a lower rate in sickle cell disease compared to normal individuals stabilising at 5 yrs of age at 5% to 8%.^[13] However some patients with sickle cell hemoglobinopathy have high levels of HbF. This includes 3 groups of sickle cell patients – sickle cell disease with Senegal/Saudi-Indian haplotype or BCL11A polymorphisms and compound heterozygous for HbS/HPFH. HbF in the former group varied between 11% to 20%, while HbF levels of 30% (SD 2) was associated with the latter group (HbS/HPFH).^[16] Further in HbS/HPFH a pan-cellular distribution of HbF is a characteristic feature. In the present study two cases showed high HbF levels of 20.65 and 32.8% along with HbS in the absence of HbA and were designated as Sickle cell disease and compound heterozygous HbS/HPFH respectively. Elevated HbF along with elevated HbA2, HbS and HbA is double heterozygous HbS/ β Thalassemia, a single case in the present study.

HbE is the second common hemoglobinopathy after sickle cell hemoglobinopathy in South-east Asia which includes North eastern India, Thailand, Malaysia, Nepal, Bangladesh and Vietnam.^[17] In the present study, HbE hemoglobinopathy accounted for 18.6% abnormal Hb similar to Sickle cell hemoglobinopathy. HbE trait was more frequent followed by HbE/ β Thalassemia and HbE disease. HbA2 levels in HbE trait was significantly higher than normal subjects (p value <0.0001) and HbA2 levels in HbE disease was significantly higher than in trait (p value $=0.035$). These findings are in corroboration with those of Mais DD who found higher HbA2 in HbE trait compared to control group and higher HbA2 in HbE homozygotes than in heterozygotes.^[6] β chain of HbE is synthesized at a reduced rate compared to HbA. Hence δ chain combines with α chain increasing HbA2 –Thalassaemic effect. Three cases of HbE hemoglobinopathy with elevated HbA2 (4.33%, SD 0.15) and HbF (11%, SD 1.0) in the absence of HbA were categorised as HbE/ β thalassemia. Since there

was no HbA these cases probably represented HbE/ β^0 thalassemia. Praising and others have similarly concluded that a combination of HbA2 levels $>6\%$ and HbF varying from 5% to 15% would differentiate HbE/ β thalassemia from HbE disease.^[18] Identifying HbE/ β Thalassemia is important since it is clinically associated with a more severe disease compared to HbE trait and HbE disease.^[17]

α Thalassemia and HbD were the less common Hb disorder in the present study similar to the other studies by Vani and others who reported 1.6% and 0.7% of these cases respectively.^[8] The predominant feature in HbH disease is the presence of HbH ranging between 0.8% to 40% with normal or slightly reduced HbA2.^[19] In the present study, HbH of 28.3% and HbA2 of 2.1% were observed. HbD common in Punjab also known as HbD-Los Angeles can be inherited in heterozygous state with HbA as in the present study and in the rarest form of homozygous state- HbDD. Association with HbS and Thalassemia also occur.^[20] MCV was lower in HbE/ β Thalassemia compared to β Thalassemia, HbE trait and SCT in the ascending order. A similar pattern has been described by Vani and others.^[8] The other Hb disorders were single cases inadequate for comparison.

Conclusion

Thus the quantification of HbF differentiates the three groups of sickle cell hemoglobinopathy patients, those of Senegal/Saudi Indian haplotype, BCL11A polymorphisms Vs heterozygous HbS/HPFH. Further the presence of elevated HbA2 and HbF in HbE hemoglobinopathy identifies HbE/ β Thalassemia, an entity needing clinical intervention. Assessment of HbA2 and HbF in conjunction with specific abnormal Hb band and HbA thus plays a pivotal role in the diagnosis of hemoglobinopathy and Thalassemia. Quantification of HbA2 and HbF thus throws light on the pattern of inheritance – homozygous/heterozygous / double heterozygous which is confirmed by molecular analysis.

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