

## HPLC: An Ideal Methodology and Screening Tool for Diagnosis of Haemoglobinopathies in Paediatric Age Group in Southern Odisha

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### Original Research Article

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**Abstract:** Haemoglobinopathies are the common genetic disorders of haemoglobin. Identification of these disorders are immensely important epidemiologically. HPLC-The bio-rad variant-II haemoglobin testing system is a totally automated Ce-Hplc-(Cation Exchange High Performance Liquid Chromatography) instrument for routine identification and quantification of normal and abnormal haemoglobin. To monitor and understand the natural history of haemoglobinopathies in southern Odisha in paediatric age group. To report the cases of rare variants of haemoglobin found in the population of Southern Districts of Odisha. To assess the accuracy and precision of diagnosis of haemoglobinopathies by HPLC and its possible advantage over conventional techniques. A total of 435 blood samples of MICROCYTIC HYPOCHROMIC ANAEMIA cases were analysed referred from different peripheral hospitals of SOUTHERN part of Odisha state for a period of two years (2015-2017). 2 ml of iv blood samples were collected after obtaining informed consent from each individual. Haematological indices were measured using automated cell counter (SYSMEX-Xt 2000i) and HPLC (BIO-RAD VARIANT-II). Background data of each individual were recorded like age, sex, caste, place of origin and consanguinity etc. Haemoglobin electrophoresis were carried out on agarose gel in Tris-EDTA-borate buffer at PH-8.9 and sickling test were carried out. Hb electrophoresis in acidic medium PH-6.2 was also carried out to confirm the presence of HbD or HbE band. Family studies were carried out for confirming the disease. Out of 435 cases 257 cases came to be positive. Sick cell disease(23.2%), Sick cell trait(17.9%), B-thalassemia trait(6.8%), Sick cell-b-thalassemia(5.2%), Beta-thalassemia major(2.9%), HPFH (0.9%), Alpha-thalassemia-(0.6%), B-thalassemia intermedia-(0.6%), Lepore-heterozygote-(0.2%), Delta-b thalassemia-(0.2%), E-B-thalassemia-(0.2%). The paediatric population is harbouring almost all major haemoglobinopathies. Sick cell anaemia has higher incidence in neonatal age group followed by sick cell trait more prevalent in childhood. HPLC is a rapid and reliable technique for identification of various haemoglobin fractions.

**Keywords:** Haemoglobinopathies, electrophoresis, HPLC.

### INTRODUCTION

The haemoglobinopathies are group of inherited diseases that are classified based on the presence of structurally abnormal haemoglobin (Hb) such as haemoglobins S, C, D and E, and/or one or more globin chain disabilities, known as thalassemsias [1]. Henderson *et al.* [2]. These pathologies are included among the most common genetic diseases in the world, with an estimated prevalence of 7% of the worldwide population [3].

It has been estimated in India, 0.37/1000 fetuses have a Hb disorder [4]. The disorders of haemoglobin frequently encountered in India are beta thalassemia, HbE-thalassemia, HbD, HbE and sickle cell anaemia. The prevalence of HbE in our country is estimated to be approximately 1.1% [5]. Worldwide Hb disorders are responsible for 3.5% mortality in children below 5 years of age [6]. World Health Organization figures estimate that 5% of the world populations are carriers of a potentially pathological haemoglobin (Hb) gene [7]. The general incidence of thalassemia trait and sickle cell haemoglobinopathy in India varies between

3-17% and 1-44% respectively but because of consanguinity and caste and area endogamy, some communities show a very high incidence, making this group of disease a major health problem in our country [8].

The Bio – Rad Variant Haemoglobin Testing system (Bio – Rad Labs, Hercules, CA), is a totally automated CE – HPLC instrument for routine quantification of HbA2, HbF and any other abnormal Hb variant. The Bio Rad variant “Beta Thalassaemia Short” program uses cation exchange HPLC to separate and elucidate the relative percentages of haemoglobin variants in whole blood. We use HPLC as a routine method for diagnosis of haemoglobinopathies in this centre. We also evaluated if this method has advantages over the conventional techniques [9].

**MATERIALS AND METHODS**

The present study was carried out over a two year period in the department of pathology MKCG medical college hospital. A total of 435 patients of anaemia with suspected haemoglobinopathies were investigated. The age group of patients ranged from 1 month to 15 years. CBC, red blood cell (RBC) indices

and peripheral blood examination were done in all cases. Sickling test was done. In all abnormal cases family studies were carried out. CE- HPLC was performed in all cases. Specimens were drawn into tubes containing dipotassium EDTA. All specimens were assessed by the Bio – Rad Variant HPLC system with the use of the Variant Beta – Thalassaemia Short Program Recorder Pack (Bio – Rad Laboratories, Hercules, CA) as described in the instrument manual for the assay. After collection, the samples were stored at 2-8°C and tested within one week of collection. Each analytical cycle, from sampling to printing of results takes about 6.5 minutes. The instrument calibration was done by loading the method parameter via a read – only memory (ROM) card provided with each set of reagent kit together with a matched set of calibrators and reagents. Care was taken to keep the RT of HbA2 as 3.65 + 0.05 minutes and total area between 10,00,000 – 30,00,000 microvolt seconds [10]. All cases showing ‘unknown peaks’ and other rare haemoglobin variants on HPLC were further analyzed by agarose gel electrophoresis at alkaline pH (8.6) and at acid pH (6.0).

**RESULTS**

**Table-1: Incidence of haemoglobinopathies**

PERIOD	CLINICALLY DIAGNOSED/SUSPECTED CASES OF HB DISORDER	HAEMATOLOGICAL LY PROVEN CASES	NORMAL STUDIES	DISEASESED (positive)	PERCENT AGE
SEP 2016- AUG 17	435	257	178(40.9%)	257	59.1%

A total of 435 cases (245 male children and 190 female children) were included in the present study. The age group of the patients ranged from 1 month to 15 years with a mean of 13.18 years and median of 12 years. Out of these, 257 cases displayed

abnormal hemoglobin fractions on HPLC. Of the total 257 cases of hemoglobin disorders, 105 were females and 152 males with a Male: Female ratio of 1.3:1. The pattern of abnormal Hb distribution observed is depicted in Table 2.

**Table-2: Spectrum of haemoglobinopathies in paediatric population**

S.NO	HAMOGLOBIN PATTERN	PATIENTS TOTAL- 435	
	HB DISORDERS	N=257	PERCENTAGE(%)
1	SICKLE CELL DISEASE	101	23.2
2	SICKLE CELL TRAIT	78	17.9
3	B-THALASSEMIA TRAIT	29	6.8
4	SICKLE-B-THALASSEMIA	23	5.2
5	B THALASSEMIA MAJOR	13	2.9
6	B-THAL INTERMEDIA	3	0.6
7	ALPHA THALASSEMIA	3	0.6
8	E-B-THALASSEMIA	1	0.2
9	DELTA-B-THALASSEMIA	1	0.2
10	LEPORE	1	0.2
11	HPFH	4	0.9
	NORMAL	178	40.9

In the present study, 101(23.2%) cases came up with sickle cell anaemia, 78(17.9%) cases had

sickle cell trait, 29 (6.8%) had b-thalassaemia trait, 23(5.2%) s-b thalassaemia, 13(2.9%) beta thalassaemia

major ,1 case of HbE -beta thalassemia(0.2%) , 4 cases had HPFH (0.93%), 3(0.6%) cases of b-thalassemia intermedia and 1 cases each of delta-b thalassemia and lepore. Remaining 178(40.9%) cases had a normal HPLC pattern. Patients heterozygous for the HbS gene showed hereditary persistence of fetal hemoglobin (HPFH).

In our study, most common abnormal haemoglobin fraction observed was HbS, seen in 179(41.1%) patients. Sickling test was positive in all of these cases.

Hb concentration in sickle cell heterozygous (AS) group ranged from 10.01±2.10 g/dL depicted in table 3.Red cell morphology was mostly normocytic normochromic. HbF was mostly normal, with 10(10.92%) patients having a raised HbF (>5.0%). In sickle cell homozygous (SS) group Hb ranged from 6.68±2.23 g/dL. Only three (3.1%) patients had Hb>11 g/dL. Most of the patients had either normocytic normochromic or microcytic hypochromic blood picture with anisopoikilocytosis. Cases were showing total leukocyte counts to be raised reason being increased nucleated RBC'S and associated infections.

**Table-3: Number, age and rbc indices of all abnormal cases**

DIAGNOSIS	NO OF CASES	AGE(YEARS)		HB(g/dl)	MCV(fl)	MCH(pg)	MCHC(%)	RDW-CV
		RANGE	MEAN					
BTI	3	0.5-8	3.4	5.37±1.78	63.9±5.2	21.67±3.88	29.02±5.46	25.97±7.48
BTM	13	0.33-10	3.44	3.83±0.90	72.52±11.5	22.28±3.95	30.52±3.52	31.10±7.13
BTT	29	0.25-15	4.7	8.32±2.67	59.7±8.4	19.16±3.17	30.24±3.82	20.21±6.00
DBT	1	0.1		7.8	67.75	21	32.5	17.5
EBT	1	5		5.80	57.90	17.10	29.40	29.40
HBH	3	1.5-13	5.5	6.7±4.2	67.8±6.9	17.40±1.55	28.05±7.00	26.40±16.12
HPFH	4	0.58-13	5.27	10.05±0.91	72.97±2.14	23.47±1.35	32.12±1.62	21.85±7.88
LEPORE	1	15		9.00	66.80	22.10	31.10	29.00
NORMAL	178	0.2-15	8.2	7.81±2.97	70.05±13.8	22.65±7.28	31.18±4.25	19.44±5.19
SB0T	1	11		5.300	87.2	28.30	32.70	22.00
SBT	22	3-15	8.7	7.73±2.63	64.81±9.0	20.61±2.0	31.20±4.00	21.14±6.45
SCA	101	0.58-15	5.4	6.68±2.36	76.06±10.10	25.09±6.11	31.37±2.95	22.09±7.75
SCT	78	0.2-15	4.9	10.01±2.10	68.51±10.45	22.34±3.70	31.79±2.86	22.08±42.93

All patients of compound heterozygous for HbS and β-thalassaemia (Sβ) were anaemic (Hb=7.73±2.63 g/dL). PBS showed moderate to marked anisopoikilocytosis. Two cases with mild anaemia were diagnosed to have compound

heterozygous for HbS and hereditary persistent of foetal haemoglobin (HPFH). Parental study of one case confirmed the diagnosis, while the other case parents did not turn around.

**Table-4: Haemoglobin fractions of all abnormal cases**

DIAGNOSIS	A2		A0		F		S	
	RANGE	MEAN±SD	RANGE	MEAN±SD	RANGE	MEAN±SD	RANGE	MEAN±SD
BTI	5.4-2.5	3.85±1.1	73-38.60	59.57 ±14.8	7.65-10.4	25.7 ±20.9	00	00
BTM	5.8-1.0	3.4±1.2	63.8-0.80	19.5 ±21.2	61.4-7.19	77.0 ±25.9	00	00
BTT	5.8-3.5	4.6±0.66	96-7.20	77.8± 20.9	1.03-4.26	9.8± 22.5	00	00
DBTHAL	4-0.80	2.4±2.2	94-45.30	69.6±34.43	29.7-25.6	27.6-±36.2	00	00
EBTHAL	60.70	60.7	00	8.1		24.20	00	00
HBH	2.7-1.7	2.2±0.70	95.290.30	92.7 ±3.46	0.18-.05	0.45± .07	00	00
HPFH	3.4-2.10	2.7±0.53	89-61.3	79.8-±13.0	4.60-9.2	12.9 ±9.2	00	00
LEPORE	10.6	10.6	00	00		90.10	00	00
NORMAL	4.0-0.60	2.5±0.41	90-29	90.16±61.68	1.03-0.39	1.81± 5.2	00	00
SB0T	4.4	4.4		2.4		11.60		80.90
SBT	5.7-2.8	4.05±0.6	63.6-1.10	27.9± 27.9	5.1-2.14	9.5± 9.8	25.7088.9	54.5 ±23.6
SCA	6.0-0.9	2.7±0.75	61.0-0.80	6.89± 11.6	18.60.708	20.0± 7.0	25.2087.9	69.9 ±11.6
SCT	3.80-2.2	3.03±2.9	82.3-43.5	56.7-±6.63	2.1-0.44	3.0± 3.8	6.80-43.1	32.66± 6.6

HbA2 levels of 3.6-9% are diagnostic of β-thalassaemia trait (BTT) in an asymptomatic individual with no or mild anaemia. We got this range of

increased HbA2 in 29 patients without any other significant abnormality in chromatogram. Most of them had microcytic hypochromic blood picture. Total

29 patients were diagnosed as BTT. 11 (22.91%) patients had Hb <9 gm% TABLE 3.

Thirteen cases were diagnosed as  $\beta$ -thalassaemia major (BTM). All presented in their 1st year of life with Hb level  $\leq 6.5$  g%. PBS showed marked degree of anisopoikilocytosis with raised RDW, hypochromia, target cells, polychromasia and NRBC. HbF levels were high. One patient had similar presentation at the age of 7 years with 81.7% of HbF levels was diagnosed as  $\beta$ -thalassaemia intermedia (BTI) on the basis of clinical data.

HbE elutes in the A2 window. We got one case of compound heterozygous for HbE and  $\beta$ -thalassaemia (E $\beta$ ) showing microcytic hypochromic picture. Hb value was 5.8 g%. The PBS showed anisopoikilocytosis with microcytosis, hypochromia and target cells. Three patient having an unknown peak

of 15.2% in the 1st minute (RT=0.39 minutes) was diagnosed as HbH disease. Presence of HbH inclusion on brilliant cresyl blue stain confirmed the diagnosis.

Hb Lepore trait constituted one case. HbA2 was raised to 10.2% with microcytic hypochromic anaemia. Majority of cases of haemoglobinopathy were from Ganjam followed by Gajapati, kandhamal and phulbani and raygada in much smaller proportions. Maximum incidence of sickle cell disease were reported from ganjam district as 56.9%. Over all incidence of thalassemia cases were again highest in ganjam district as 78.5% followed by kandhamal (9.5%) followed by gajapati district (7.14%) and least recorded in phulbani and rayagada areas. sickle cell thalassemia scored highest in occurrence in ganjam district with 82.6% followed by gajapati and kandhamal Table 5.

**Table 5: District-wise distribution of sca, thalassemia disease**

DISTRICT	SICKLE CELL DISEASE		THALASSEMIA		SICKLE CELL HALASSEMIA	
Gajapati	64	35.7%	03	7.14%	02	8.6%
Ganjam	102	56.9%	33	78.5%	19	82.6%
Kandhamal	07	3.9%	04	9.5%	02	8.6%
Phulbani	03	1.6%	01	2.3%	00	0.0%
rayagada	03	1.6%	01	2.3%	00	0.0%
Total	179		42		23	

**DISCUSSION**

India is an ethnically diverse country with marked regional variation. This diversity is reflected in the presence of different haemoglobin variants in different ethnic groups. Moreover, due to migration, there is constant mixing of people from different regions. Many of these abnormal variants are of little clinical significance in heterozygous state, but when combined with other variants they may give rise to severe disease, especially the compound heterozygous disorders (HbSD – Punjab, HbSE, HbS -  $\beta$  thalassaemia) or unusual variants (HbQ India, HbD Punjab, HbD Iran), are all clinically significant with varying degrees of severity, making precise identification important [11,12].

The haemoglobinopathies and thalassemia are the most common inherited single gene disorder in India. Therefore there is always a need for a screening method which can detect maximum variants. The identification of haemoglobin variants by conventional techniques are often presumptive, based on the electrophoretic mobility of the band, quantification and/or ethnic origin of the parents. HPLC has the advantage of quantifying HbF and HbA2 along with detecting other variants in a single screening test.

In any given population, it is the children that are both most vulnerable as well as most suitable for timely intervention and efficacious treatment. It is also this paediatric age group that genuinely reflects the challenges we are facing as a society from hemoglobinopathies.

In the present study, out of 435 clinically suspected cases, the prevalence of Hb disorders was found to be 59.5%. This is much higher than 31 cases out of 105 (29.5%) in the series of cases studied by Mahapatra *et al.* [13] in Odisha. Previous two institutional based studies from Odisha reported prevalence of 44.2% and 65.7% of Hb disorders [14,15]. Sarojini *et al.* [16] (KIIMS ODISHA) in their study came up with 37.18% of abnormal haemoglobin fractions which is again lower in prevalence as compared with our study.

However, there is a wide range of variation in prevalence of Hb disorders in different parts of India. Madan et al reported a prevalence of 11.43% in a study from West Bengal [17]. Two hospital based studies from Western India got 8.6% and 11.36% of abnormal haemoglobin variants [18,19]. In the North Indian population, incidence of haemoglobinopathies was found to be 12.5% [20]. Baruah *et al.* noted a high

prevalence (59.11%) equal to southern Odisha in North-East region [21].

HbS was detected in 41.1% patients, which was slightly higher than reported by Balgir *et al.* (39.3%)[14], Sarojini *et al.* [16]. (28.17%) and Dash *et al.* (29.0%) [15] From Odisha.

Most common abnormal Hb pattern detected in this study was sickle cell anaemia (SA) as 23.21% followed by AS (17.90%) and BTT (6.6%). The two above said studies from Odisha found AS to be the most common variant followed by SS and BTT [14,15]. However, most of the other studies across the country reported BTT as the most common variant [17,18,22,19,20,23,24].

As this was a hospital based study which depends upon presenting complaints and signs and symptoms, number of children affected with sickle cell disease catastrophe outnumbered other haemoglobin disorders and thalassemia cases in this study. In contrast, population based studies would show the actual prevalence of various Hb disorders. In spite of these variations, the high incidence of both AS and BTT highlights the need for antenatal screening for prevention of more severe form like BTM, SS and S $\beta$  in offspring.

The present study also showed a microcytic hypochromic blood picture in AS group, which could be due to associated iron deficiency. High incidence of iron deficiency has been reported in patients with sickle cell disease from India as reported by Balgir RS *et al.*[25] and Sarojini raman *et al.*[16].

The finding of a raised HbF in some sickle cell trait patients and normal population was difficult to explain showing increased HbF levels in this region. Many sickle cell homozygous patients had HbF >30%. Agarwal *et al.* said that in Indian subcontinent HbSS patients has slightly higher HbF level, than the other parts of the world. The reason for this is that the haplotype of HbS gene, which is prevalent in India, is the Saudi Arabia/Indian haplotype, that reduces the clinical severity of the disease [18,26].

BTT was the 3rd most common abnormal haemoglobin variant we got which was in concordance with the study conducted by Sarojini *et al.*[16]2017. Most of the cases had characteristic microcytic hypochromic red cells with normal or slightly reduced Hb and raised RBC count. Raised HbA2 level is the most important abnormal chromatogram finding helpful for its diagnosis.

However, conditions with borderline HbA2 need careful interpretation. Nutritional anaemia must always be taken into account. A low level of HbA2 may be induced by iron deficiency. Similarly,

cobalamine or folate deficiency may raise HbA2 level. However, Rao s *et al.* study shows no significant difference in HbA2 level in patients of BTT with or without concomitant iron deficiency. Thus elevation in HbA2 level can be used with reliability for diagnosis of BTT even in the presence of iron deficiency [27,28].

In the present study, the peripheral red cell morphology was of help in cases of borderline elevation of HbA2. In cases of cobalamine and folate deficiency repeat HPLC were advised after nutritional supplement whenever feasible. Cases with normal A2 level on repeat HPLC were taken as normal and cases still having raised A2 were diagnosed as BTT. Similarly, milder forms of thalassemia or a co-inheritance of delta thalassaemia may also lead to borderline A2 levels. Genetic studies should be hence advised in cases of dilemma for a conclusive opinion [20].

BTM was seen in 13(5%) patients. This was in concordance with the studies conducted by J singh *et al.* 2016[24] (4%), Rao s *et al.* (2.9%) [27].

In 2008 patel j *et al.* stated Thalassaemia intermedia is suspected when a patient presents after 3 years of age or needs fewer blood transfusion [29]. One of our patients presented like major at the age of 7 years and diagnosed as BTI but remaining two cases presented at 1-2 years of age.

In our study, the prevalence of HbE gene was 0.22% (1 case of E-B thalassaemia) which is slightly lower than the finding observed by Sarojini *et al.* 2017(1.63%) and Balgir *et al.* (1.90%) in the other regions of Odisha[14,16].

HbE is the most frequent variant Hb in Asia, with a significant prevalence in North-East India and Bangladesh. It is a  $\beta$ -chain variant that tends to elute in A2 window on HPLC. HbE homozygous usually presents with HbE values >70-75% and heterozygous with HbE values <40% [20]. E $\beta$  (1) case in present study had severe anaemia (Hb $\leq$ 6.13 g%) with HbA2 =60%. The comparatively high value of HbA2 is explained by the presence of A2 component of beta thalassaemia, justifying the criteria set for HbE hetrozygous A2 levels. Biswas ak *et al.* [18] said Clinical effects are more severe when HbE is coinherited with  $\beta$ -thalassaemia (E $\beta$ )[18,20].

Though HPFH homozygous cases have been reported, we could not find much literature about it. We diagnosed four cases of mild anaemia as hereditary persistence of fetal haemoglobin based on raised HbF with normal HbA and HbA2. Parental study confirmed the diagnosis in one case. Molecular study was advised in the other cases for confirmation.

Other variants detected in the present study included one case of Hb lepore (0.2%) and three cases of HbH (0.9%). Hb Lepore also elutes in A2 window. A2 level in the case of Hb lepore in the present study was 10.8%. Like Hb Lepore, Hb D Iran also elutes in A2 window. A sharp peak in the first minute of elution indicates HbH [30]. HbH disease shows considerable variability in clinical and haematological severity.

The present study included 130 cases of sickle cell disorder and thalassemia with organomegaly and one case of BTM with typical hemolytic facies. In the peripheral smear all the cases showed the classical findings but the MCV, MCH values were not correlating with the degree of anemia. Also in some cases of heterozygous condition (carrier), peripheral smear showed no abnormality except for mild microcytic anemia in few cases. So, to detect a carrier status, HPLC is essential.

In the present study, history of consanguinous marriage found in 14 cases was the predominant cause for the occurrence of double heterozygous cases. 19 cases of double heterozygous was reported by Patel D.K *et al.* [31] and 24 by Tariq H.A *et al.* [32] stating that the main reason for increased incidence of double heterozygous cases in particular communities like Scheduled caste and Muslims is consanguinity.

Colah R *et al.* stated that thalassaemic individuals have a reduced MCV, and other studies have suggested that an MCV of <72 is maximally sensitive and specific for the presumptive diagnosis of thalassemia [33, 34]. As per the study by Chopra b s nair *et al.* low MCH and MCV are the clues for the diagnosis of thalassemia [35].

It is denoted that thalassemia carriers have characteristic hematological parameters with mild or no anemia with microcytic hypochromic RBCs, increased red cell counts and normal RDW, and that hemoglobinopathies can be suspected on the basis of these parameters. This needs to be differentiated from other causes of microcytic hypochromic anemia like iron deficiency anemia. RDW measures the coefficient of variation and is higher in iron deficiency anemia but not in thalassemia, where a uniform microcytic red cell population is seen with a normal RDW. The mean red blood cell (RBC) count in carriers of hemoglobinopathies was increased and considered a useful diagnostic adjunct because thalassemia patients have microcytic anemia with an increase in the RBC number, whereas other causes of microcytic anemia, including iron deficiency anemia and anemia of chronic disease, are typically associated with a decrease in the RBC number that is proportional to the degree of decrease in Hb concentration [36].

In the present study eight patients, who were suspected to have associated IDA and borderline HbA2

levels with HbA2 values >3.9%, were advised for re-evaluation on HPLC after iron therapy. 3 of them showed normal A2 values and were included in normal group. 5 cases with persistent raised HbA2 (>4%) were diagnosed as BTT. One patient was lost to follow up, were excluded from the study. In a study by madan *et al.* a significant decrease in HbA2 levels in thalassemia trait patients are associated with IDA[181,182]. Apart from IDA, Megaloblastic anaemia will also result in borderline HbA2 levels, the latter returning to normal after adequate therapy [37-41].

Analysis of geographical distribution shows that maximum incidence of haemoglobinopathies were noted in the district of Ganjam (39.6%), which can be explained by this hospital being located in the centre of the district and catering to the whole of Ganjam district. Next districts were Gajapati (31.5%) and Kandhamal(18.5%), followed by Phulbani(5.85%) and Rayagda(5.05%) showing least incidences. In a study by Mahapatra *et al.* [13], maximum incidence of sickle cell cases was seen in Dhenkanal followed by Cuttack and thalassemia was maximum in Balasore and Cuttack district, all of them belonging to coastal belts of Orissa.

Amongst haemoglobin disorders in different subdivisions of Ganjam, the maximum incidence was of sickle cell haemoglobinopathy with 31 cases (30.7%) followed by sickle cell thalassemia 10 cases (52.6%) and thalassemia 21 cases (63.6%) were from Berhampur, followed by hilly subdivision of Bhanjanagar followed by lower incidence in Chatrapur and Aska subdivisions. From this study it was evident that the sickle cell haemoglobinopathies and thalassemia's are prevalent in coastal as well as non-coastal areas of southern Orissa with more prevalence in the hilly tracts.

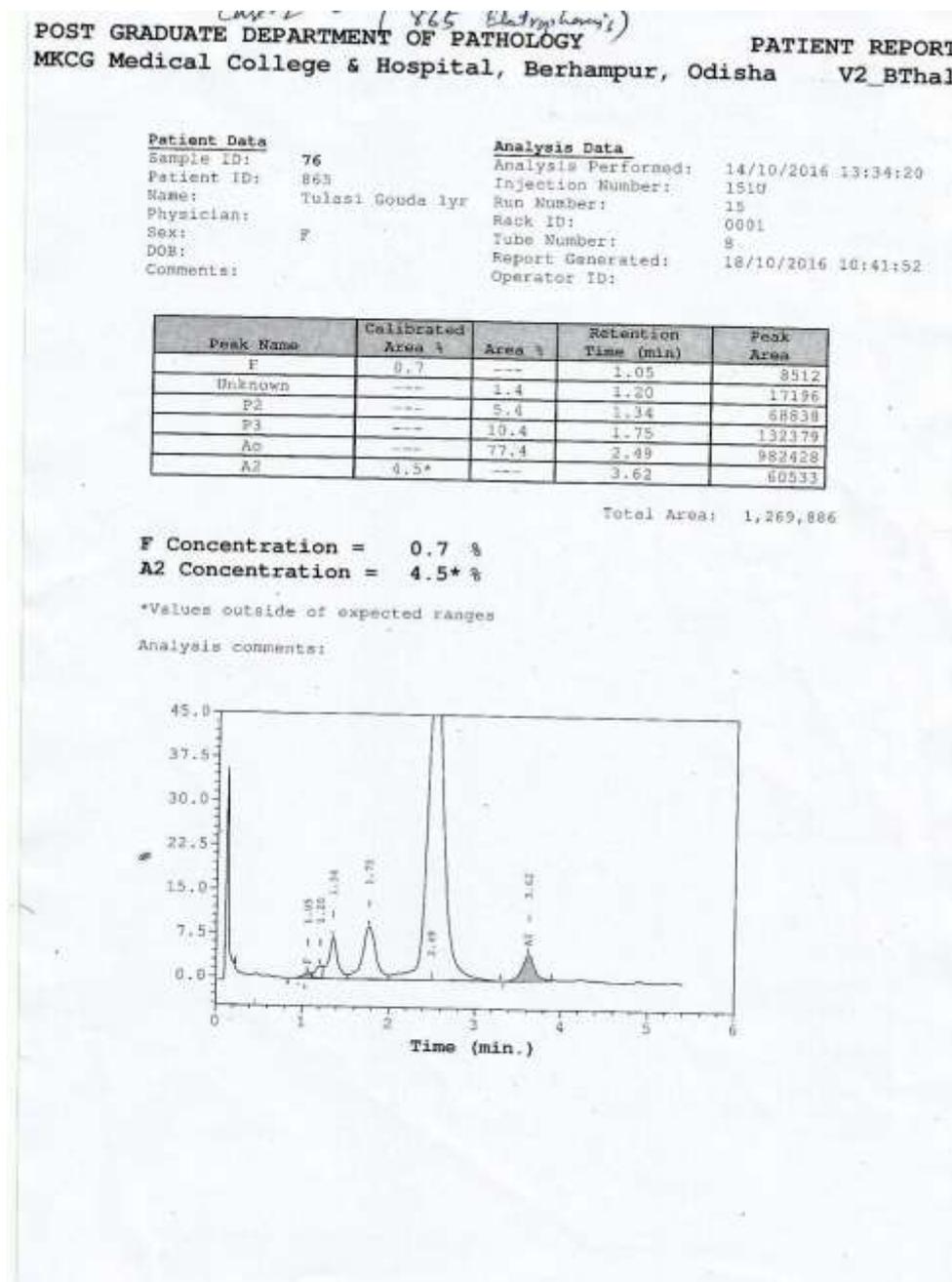
Although Hb electrophoretic technique has several advantage like its being very cost effective, HPLC techniques score over electrophoresis in many aspects [42]. It is a rapid method for quantitative haemoglobin analysis. This technique is uncomplicated and helpful in identifying haemoglobins which have the same mobility on electrophoresis and can run multiple batches of sample in a small span of time.

Although HbE and HbS syndromes could be diagnosed by both HPLC and gel electrophoresis, the use of HPLC helped in further sub – characterization of these syndromes based on quantification of HbE, HbS and HbA2 levels. Use of HPLC, also helped in easier diagnosis of one cases each of compound heterozygous sickles -  $\beta^+$  thalassaemia, E-b thalassemia and Delta-b thalassemia. Amongst the patients who had Hb band at SDGLepore region in gel electrophoresis and negative sickling test, one patient was diagnosed as Hb lepore trait on HPLC.

It is thus recommended that in all cases where Hb migration occurs in SDGLepore region on gel electrophoresis, HPLC should definitely be performed for further sub characterization of rare Hb variants [11].

In conclusion, the simplicity of sample preparation, accurate quantification of haemoglobin concentration combined with complete automation, makes HPLC an ideal methodology for the routine diagnosis of haemoglobin disorders.

4(a) Alpha thalassemia (HbH ds)



HPLC-CHROMATOGRAMS-4(a) to 4(i)

POST GRADUATE DEPARTMENT OF PATHOLOGY PATIENT REPORT  
 MKCG Medical College & Hospital, Berhampur, Odisha V2\_BThal

Case-1

<b>Patient Data</b>		<b>Analysis Data</b>	
Sample ID:	73	Analysis Performed:	14/10/2016 13:14:37
Patient ID:	862	Injection Number:	1480
Name:	Sital das priyada	Batch Number:	15
Physician:	EMT	Back ID:	0001
Sex:	F	Tube Number:	5
DOB:	14/10/2016	Report Generated:	10/10/2016 10:39:10
Comments:	SICKLE CELL ANEMIA CONFIRMED		

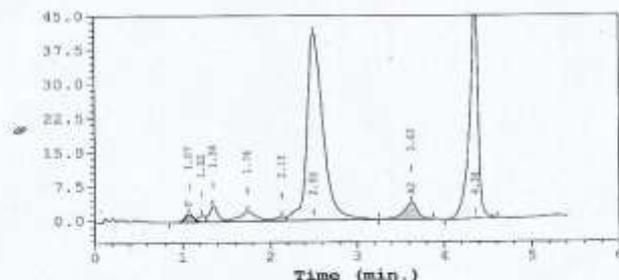
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	1.2	---	1.07	20902
Unknown	---	0.8	1.22	14844
P2	---	2.3	1.34	41912
P3	---	2.7	1.74	49404
Unknown	---	0.7	2.13	12803
A1	---	54.3	2.50	977883
A2	3.7*	---	3.62	70222
S-window	---	34.0	4.36	612021

Total Area: 1,800,290

F Concentration = 1.2 %  
 A2 Concentration = 3.7\* %

\*Values outside of expected ranges

Analysis comments:



4(b) sickle cell anaemia heterozygous

POST GRADUATE DEPARTMENT OF PATHOLOGY PATIENT REPORT  
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Case-3

<b>Patient Data</b>		<b>Analysis Data</b>	
Sample ID:	75	Analysis Performed:	14/10/2016 13:27:42
Patient ID:	864	Injection Number:	150W
Name:	Chhabita Pradhan 19yr	Run Number:	15
Physician:	Paed	Rack ID:	0001
Sex:	F	Tube Number:	7
DOB:		Report Generated:	14/10/2016 10:40:40
Comments:		Operator ID:	

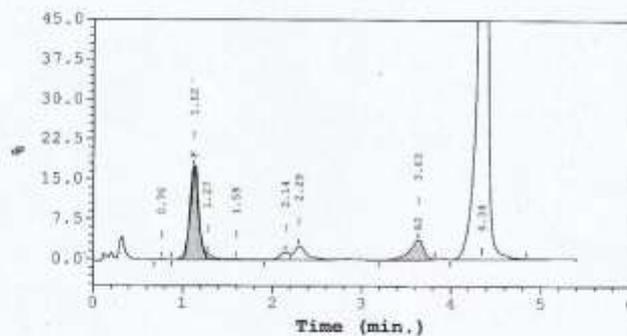
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.0	0.76	727
F	11.5*	---	1.12	212662
P2	---	0.6	1.27	11216
P3	---	0.1	1.59	3383
Unknown	---	1.1	2.14	20292
Ao	---	2.4	2.29	46122
A2	3.7*	---	3.63	74818
S-window	---	80.8	4.34	1545404

Total Area: 1,912,624

**F Concentration = 11.5\* %**  
**A2 Concentration = 3.7\* %**

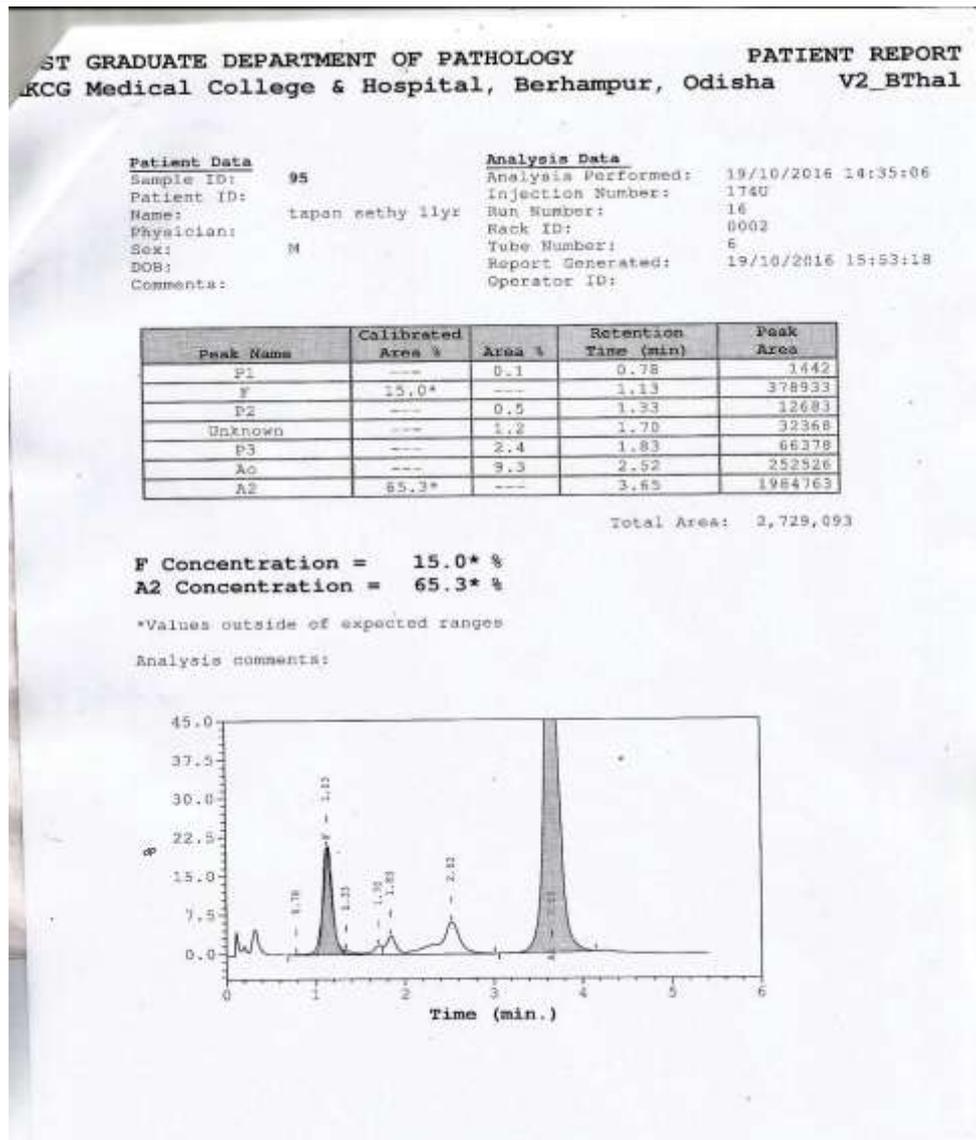
\*Values outside of expected ranges

Analysis comments:



4(c) sickle cell anaemia homozygous





4(e) compound heterozygous for HbE and B-thalassemia

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<b>Patient Data</b>		<b>Analysis Data</b>	
Sample ID:	31	Analysis Performed:	28/09/2016 14:01:46
Patient ID:	809	Injection Number:	780
Name:	sampurna sahak	Run Number:	8
Physician:		Peak ID:	0002
Sex:	M	Tube Number:	2
DOB:	28/09/2016	Report Generated:	18/10/2016 10:44:57
Comments:		Operator ID:	

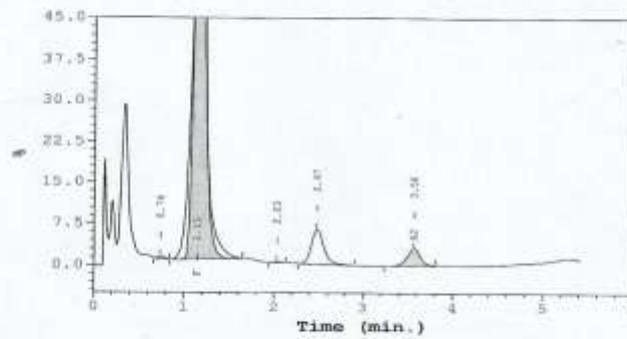
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F1	---	0.2	0.74	2418
F	94.4*	---	1.15	905721
Unknown	---	0.1	2.03	591
A0	---	6.5	2.47	65636
A2	3.2	---	3.56	34638

Total Area: 1,009,404

F Concentration = 94.4\* %  
 A2 Concentration = 3.2 %

\*Values outside of expected ranges

Analysis comments:



4(f) beta thalassemia major

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**MKCG Medical College & Hospital, Berhampur, Odisha**      **V2\_BThal**

<p><b>Patient Data</b></p> <p>Sample ID: 4                  Patient ID: 780                  Name: SRABANI NAYAK                  Physician:                  Sex: F                  DOB: 23/09/2016                  Comments:</p>	<p><b>Analysis Data</b></p> <p>Analysis Performed: 23/09/2016 15:25:14                  Injection Number: 680                  Run Number: 7                  Rack ID: 0004                  Tube Number: 4                  Report Generated: 23/09/2016 15:55:19                  Operator ID:</p>
--	--

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.7	---	1.06	10351
Unknown	---	0.7	1.26	10413
P2	---	2.0	1.33	31197
P3	---	4.5	1.73	69498
Ao	---	54.8	2.49	840263
A2	4.4*	---	3.59	70284
S-window	---	32.6	4.35	499959

Total Area: 1,531,966

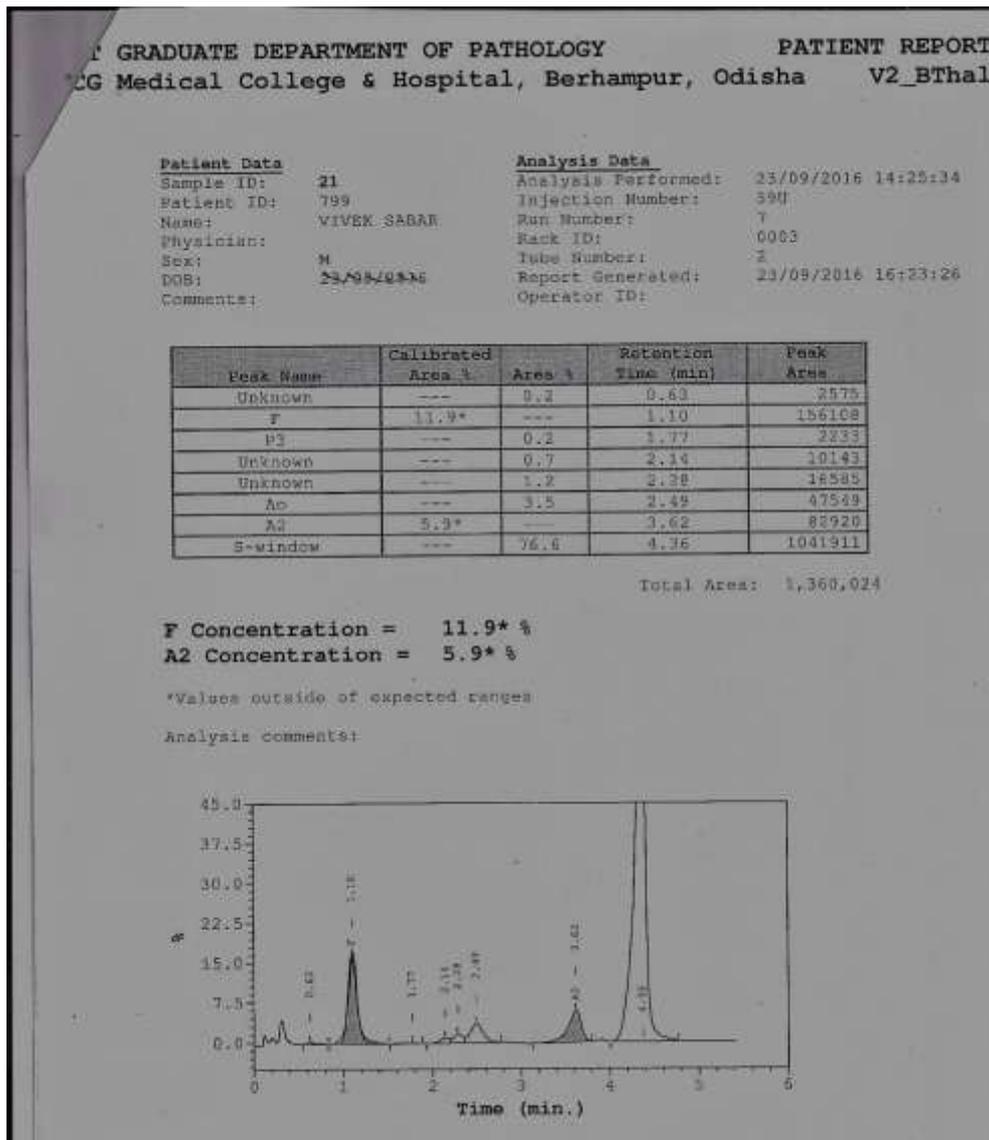
**F Concentration = 0.7 %**  
**A2 Concentration = 4.4\* %**

\*Values outside of expected ranges

Analysis comments:

*Report*

**4(g) Hbs -b<sup>+</sup>-thalassemia**



4(h) Sickle-b0 thalassemia

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	8.0*	---	1.14	173301
P2	---	5.1	1.30	111626
P3	---	4.1	1.69	88592
Ao	---	80.1	2.46	1743633
A2	2.8	---	3.61	58347

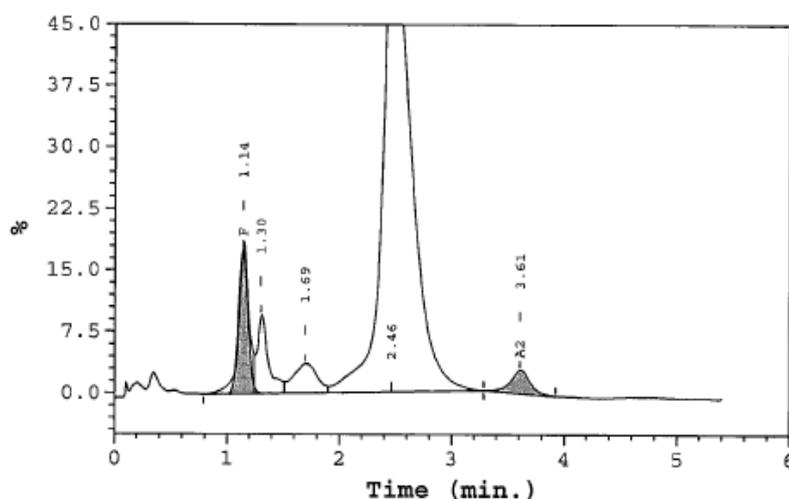
Total Area: 2,175,500

F Concentration = 8.0\* %

A2 Concentration = 2.8 %

\*Values outside of expected ranges

Analysis comments:

4(i) delta - $\beta$  thalassemia

**Fig-4: Chromatogram of (a) alpha thalassemia(HBH), (b) Sickle Cell Heterozygous (SA), (c) sickle cell anemia homozygous(SS), (d) beta thalassemia trait, (e) Compound Heterozygous for HbE and  $\beta$ Thalassaemia (E $\beta$ ), (f)  $\beta$ -thalassaemia Major (BTM), (g) HbS -b+ Thalassaemia, (h) HbS-b0 thalassemia, (i) delta-beta thalassemia**

## CONCLUSION

Haemoglobinopathies form a significant proportion of hereditary disorders in paediatric population leading to a range of myriad complication, leading to mortality in large number of afflicted patients. Most common forms of these include thalassaemia and sickle cell anaemia. Published literature includes various reports on screening patient using HPLC in adults as well as paediatric population. However, there is paucity of literature on studies on exclusive paediatric population. In India, where  $\beta$ -thalassaemia trait is so rampant, premarital and antenatal screening should be mandatory to prevent birth of children with  $\beta$ -thalassaemia major. The simplicity and rapidity of sample preparation, accurate quantification of Hb concentration combined with complete automation, makes HPLC an ideal methodology for the routine diagnosis of Hb disorders.

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