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THE STUDY OF DOUBLE HETEROZYGOSITY BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) IN HEMOGLOBIN DISORDER : A CLINICOHEMATOLOGICAL CORRELATION

Medical Science

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ABSTRACT

Introduction: The inherited disorders of blood include hemoglobinopathies as one of the major public health problems in India.They cause a high degree of morbidity, moderate to severe hemolytic anemia among vulnerable segments of the society like infants and children, adolescent girls, pregnant women, etc. and several deaths in India.The cumulative gene frequency of the three most predominant abnormal hemoglobins, i.e. sickle hemoglobin, hemoglobin D and hemoglobin E has been found to be 5.35% in India. Prevalence of sickle gene is found to be 0-18% in North eastern India, 0-33.5% in Western India, 22.5-44.4% in Central India and 1-40% in Southern India. Average prevalence of sickle cell carrier among the tribal population was 10%. The sickling disorders- HbSS, HbSD, HbSE, HbS/ β- thalassemia and other compound heterozygous hemoglobinopathies are all clinically significant, as these combinations present with different manifestations and degrees of severity, making precise identification important. Automated cation-exchange High Performance Liquid Chromatography (HPLC) has emerged as an excellent screening tool for diagnosing these abnormal hemoglobins/ thalassemic states.

Aims and Objective: 1) To study clinical features, hematological features and HPLC findings in patients of compound heterozygous hemoglobin disorders. 2) To generate data regarding ethnicity and consanguinity in cases of compound heterozygous hemoglobin disorders.3) To evaluate the role of family study in cases of compound heterozygous hemoglobin disorders.

Material and Methods: This is a study of double heterozygosity by High Performance Liquid Chromatography (HPLC). The study was carried out in Department of Pathology, RIMS, Ranchi, after the approval of the college ethical committee. Study design: The present study is a Cross sectional descriptive study. Study period: Conducted from July 2013 to August 2014.

Observation and result: In the present study, total 70 cases of compound/ double heterozygous state were studied in the Department of Pathology, Rajendra Institute of Medical Sciences, Ranchi from July 2013 to August 2014. The compound heterozygous states are grouped as Group A- Sβ thalassemia - 22+38=60 cases (85.71%), Group B- Eβ thalassemia - 6+4=10 cases (14.28%). 54.28% cases showed consanguinity. This highlights the role of premarital counseling. Scheduled Tribe (ST) (37.14%) and Muslim (27.14%) were the most commonly affected ethnic groups, this emphasizes on the need to formulate community based studies and implement on the strategies accordingly. Pallor was the most common feature among all the groups followed by jaundice and splenomegaly. Anemia, jaundice and splenomegaly should be investigated in detail for the possibility of Hb disorder.

Conclusion: The definite identification of disorders of hemoglobin synthesis can be achieved only by DNA analysis, but, in the Indian scenario, family studies on HPLC might be useful as there is paucity of funds, and facilities for DNA analysis are not readily available. Family study is an equally efficacious and cost effective tool. This study is carried out to correctly diagnose the compound heterozygous states by performing HPLC and family studies if possible, and to generate data so that health care resources can be successfully planed and targeted at them.

KEYWORDS

HPLC, Double heterozygous, Sickle beta thalassemia, E beta thalassemia, Hemoglobinopathies.

INTRODUCTION :

The inherited disorders of blood include hemoglobinopathies as one of the major public health problems in India.

They cause a high degree of morbidity, moderate to severe hemolytic anemia among vulnerable segments of the society like infants and children, adolescent girls, pregnant women, etc. and several deaths in India.

The cumulative gene frequency of the three most predominant abnormal hemoglobins, i.e. sickle hemoglobin, hemoglobin D and hemoglobin E has been found to be 5.35% in India.[1]

WHO figures about 5% of the world's population carries genes responsible for hemoglobinopathies. Each year about 3, 00,000 infants are born with major hemoglobin disorders- including more than 2, 00,000 cases of sickle cell anemia in Africa. It primarily affects people of African, Mediterranean, Middle Eastern and Asian Indian ancestry.[2] Prevalence of sickle gene is found to be 0-18% in North eastern India, 0-33.5% in Western India, 22.5-44.4% in Central India and 1-40% in Southern India. In our country, tribals with sickle gene are mainly concentrated in Madhya Pradesh, Orissa, Chattisgarh, Jharkhad, Gujrath, Andhra Pradesh etc.[3]

The hemoglobin sickle (HbS) syndrome is frequently seen in various parts of India. It includes- Sickle cell disease, sickle cell trait, sickle cell β thalassemia.[4]

The β thalassemias and their interaction with structural hemoglobin variants like HbS and HbE are a major public health problem in India.[5]

The sickling disorders- HbSS, HbSD, HbSE, HbS/ β- thalassemia and other compound heterozygous hemoglobinopathies are all clinically significant, as these combinations present with different manifestations and degrees of severity, making precise identification important.[6]

There is wide variation in the longevity and clinical pattern of sickle cell disorders in different communities. The recognized factors that tend to affect the variability include age, concomitant presence of thalassemia gene, high level of fetal hemoglobin, presence of glucose-6-phospate dehydrogenase deficiency and environmental factors like infections, dehydration, extremes of temperature, acidosis, hypoxia and emotional stress.[7]

Automated cation-exchange High Performance Liquid Chromatography (HPLC) has emerged as an excellent screening tool for diagnosing these abnormal hemoglobins/ thalassemic states.[6]

The retention time on HPLC is reliable, reproducible, and in many cases superior to conventional hemoglobin electrophoresis for the detection and identification of hemoglobin variants.[8]

Hemoglobin fraction analysis by cation-exchange HPLC has the advantage of quantifying HbF and HbA_2 along with hemoglobin variant screening in a single, highly reproducible system, making it an excellent technology to screen for hemoglobin variants and hemoglobinopathies along with the thalassemias. The simplicity of the automated system with internal sample preparation, superior

resolution, rapid assay time, and accurate quantification of hemoglobin fractions makes this an ideal methodology for the routine clinical laboratory.[8]

The definite identification of disorders of hemoglobin synthesis can be achieved only by DNA analysis, but, in the Indian scenario, family studies on HPLC might be useful as there is paucity of funds, and facilities for DNA analysis are not readily available. Family study is an equally efficacious and cost effective tool.[6]

It is possible to provide a better quality of life, and, in some cases, a definitive cure for patients with sickle cell disorder. However, these advances, which are mainly applicable in high resource countries, have unfortunately widened the gap in terms of quality of life between patients in developed countries and those in developing countries, and that gap can be reduced only through epidemiological studies, an improvement in health services, health education.[2]

This study is carried out to correctly diagnose the compound heterozygous states by performing HPLC and family studies if possible, and to generate data so that health care resources can be successfully planed and targeted at them.

MATERIAL AND METHODS :

The study was carried out in Department of Pathology, Rajendra Institute of Medical Sciences, Ranchi, Jharkhand, India.

Inclusion criteria: All the patients of anemia (OPD and Indoor) showing sickling test positive,Patients presenting with hepatosple nomegaly, Patients with clinical suspicion of hemolytic anemia, Family members of these patients.

Exclusion criteria: Patients who received blood transfusion in the last 6 months were excluded from the study.

Plan of the Study: After screening consecutive cases as per the above mentioned criteria, detailed history was taken and complete clinical examination was done.

After consent from parents, 4 ml of venous blood was withdrawn from patients and collected in EDTA (Ethylene diamine tetra-acetic acid) anticoagulant bulb and studied for HPLC curves and complete blood count (CBC).

The blood sample were run in 5 part Sysmax hemato-analyzer before performing HPLC (All consumable items, including reagents, wash solution and mini cartridges, were from Bio-Rad. Beta thalassemia short program was used in the study) to obtain the haemoglobin values and indices.

Then study of family members accompanying the cases was done to confirm the diagnosis and to determine ethnic background.

Table no 1: Analysate Identification Windows:

Retention time (minutes)	Band (minutes)	Window (minutes)	Range
F	1.15	0.15	1.00-1.30
P2	1.45	0.15	1.30-1.60
P3	1.75	0.15	1.60-1.90
A0	2.60	0.40	2.20-3.30
A2	3.83	0.15	3.68-3.98
D-window	4.05	0.15	3.98-4.12
S-window	4.27	0.15	4.12-4.42
C-window	5.03	0.15	4.88-5.18

P2 and P3 are minor peaks associate with glycosylated HbA.
D window – HbD Punjab elutes.

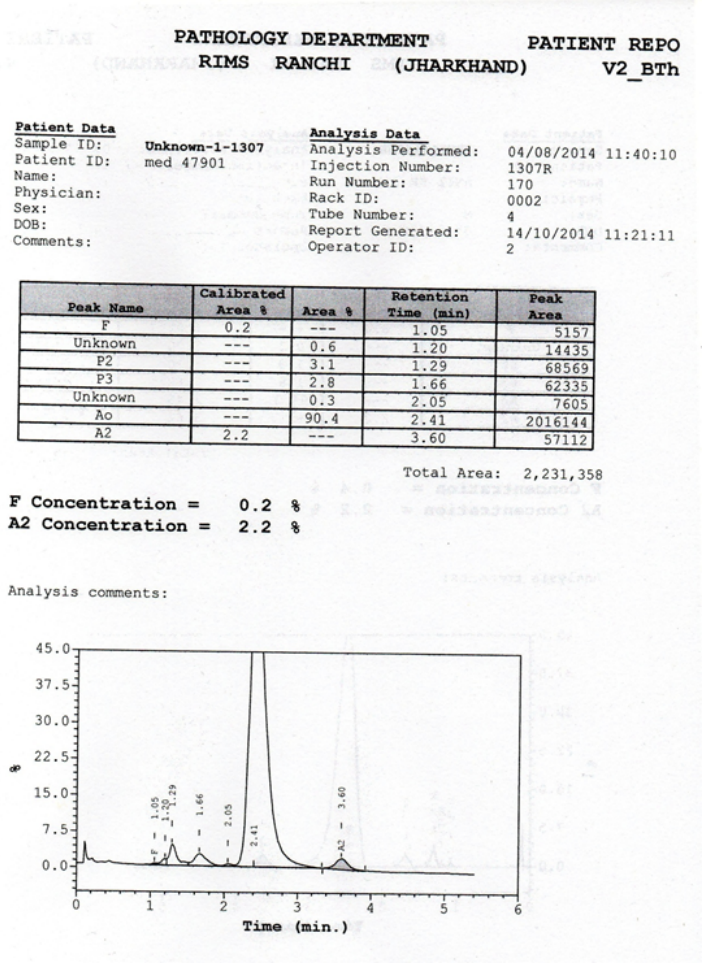
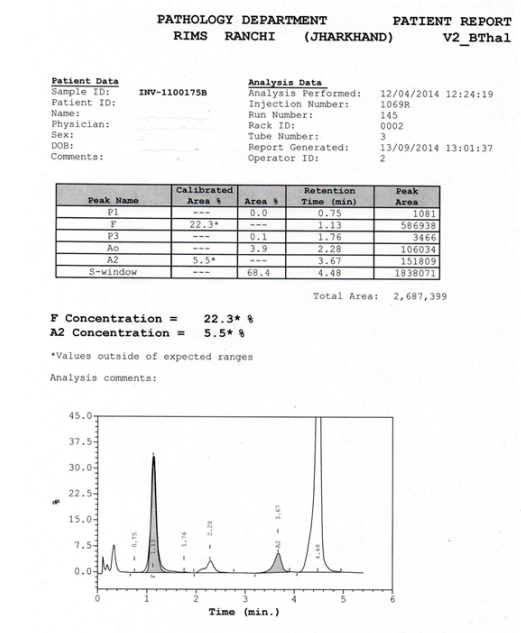


Fig.1: Normal pattern.



PATHOLOGY DEPARTMENT PATIENT REPORT
RIMS RANCHI (JHARKHAND) V2_BThal

Patient Data
Sample ID: INV-1100175B
Patient ID:
Name:
Physician:
Sex:
DOB:
Comments:

Analysis Data
Analysis Performed: 12/04/2014 12:24:19
Injection Number: 1069R
Run Number: 145
Rack ID: 0002
Tube Number: 3
Report Generated: 13/09/2014 13:01:37
Operator ID: 2

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.0	0.75	1081
F	22.3*	---	1.13	586938
P3	---	0.1	1.76	3466
Ao	---	3.9	2.28	106034
A2	5.5*	---	3.67	151809
S-window	---	68.4	4.48	1838071

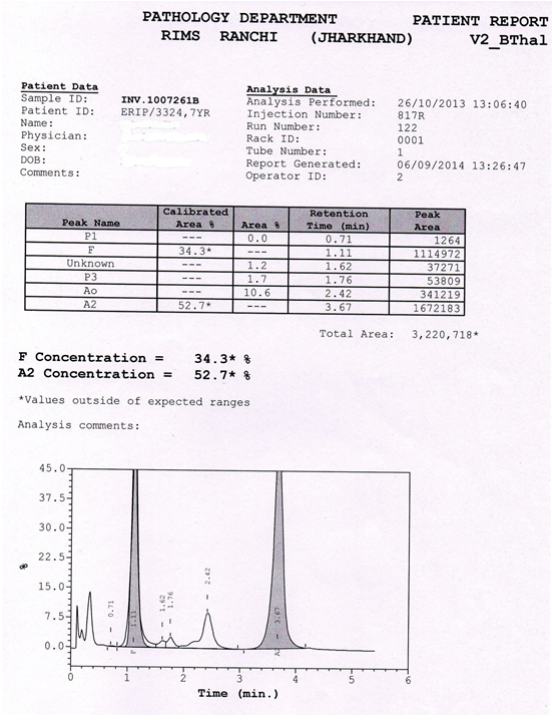
Total Area: 2,687,399

F Concentration = 22.3* %
A2 Concentration = 5.5* %

*Values outside of expected ranges

Analysis comments:

Fig.2: Sβ thalassemia



PATHOLOGY DEPARTMENT PATIENT REPORT
RIMS RANCHI (JHARKHAND) V2_BThal

Patient Data
Sample ID: INV.1007261B
Patient ID: ERIP/3324,7YR
Name:
Physician:
Sex:
DOB:
Comments:

Analysis Data
Analysis Performed: 26/10/2013 13:06:40
Injection Number: 817R
Run Number: 122
Rack ID: 0001
Tube Number: 1
Report Generated: 06/09/2014 13:26:47
Operator ID: 2

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.0	0.71	1264
F	34.3*	---	1.11	1114972
Unknown	---	1.2	1.62	37271
P3	---	1.7	1.76	53809
Ao	---	10.6	2.42	341219
A2	52.7*	---	3.67	1672183

Total Area: 3,220,718*

F Concentration = 34.3* %
A2 Concentration = 52.7* %

*Values outside of expected ranges

Analysis comments:

Fig.3: Eβ thalassemia

OBSERVATION AND RESULT :

In the present study, total 70 cases of compound/ double heterozygous state were studied in the Department of Pathology, Rajendra Institute of Medical Sciences, Ranchi from July 2013 to August 2014.

Table no.2: Cases are grouped as:

Group	Gr-A Sickle-Beta Thalassemia	Gr-B HbE-Beta Thalassemia	Total
No. of cases	22+38* Total cases 60 (85.71%)	6+4** (14.28%)	70

In Sickle-Beta Thalassemia 22 cases-Confirmed by family study; *38 cases-Provisionally diagnosed without family study {HbA2 >4 %} [4,14]
In HbE-Beta Thalassemia 6 cases confirmed by family study; ** 4 cases- Provisionally diagnosed without family study.

So, by complete hematological work up, HPLC study and family study cases are classified as:

Group A: Sickle-Beta Thalassemia- (Sβ Thal.)
Group B: HbE-Beta Thalassemia- (Eβ Thal).
Reasons for incomplete family study: Family members' samples not accompanied at the outset of the work up due to:

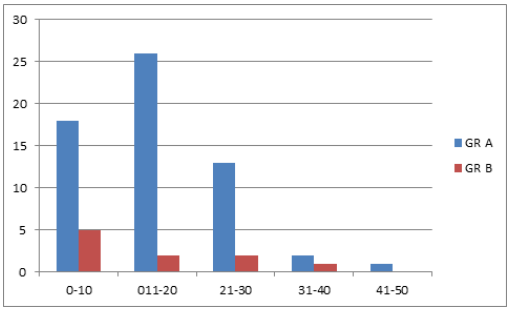
Distance	Remarriage/Death
Poor socio-economic status	Reluctance (esp. in father)
Lack of awareness/ Misconceptions	Alcoholism/ Psychosocial reasons

Table no.3 : Age and sex distribution among all the groups-

Age (years)	Gr-A Sβ thal n=60				Gr-B Eβ thal n=10			
	M	F	T	%	M	F	T	%
0 to 10	14	4	18	30	3	2	5	50
11 to 20	16	10	26	43.33	1	1	2	20
21 to 30	5	8	13	21.66	1	1	2	20
31 to 40	-	2	2	3.33	1	-	1	10
41 to 50	1	-	1	1.66	-	-	0	-

M=Male , F=Female, T=Total

AGE DISTRIBUTION:



In Group A, i.e., Sβ thal:
Maximum cases were in 11-20 years age group.
In Group B, i.e., Eβ thal:
Maximum cases were in 0-10 years age group.

Table no.4 : History of consanguineous marriage:

Group	Gr-A Sβ thal	Gr-B Eβ thal	Total n=70	%
H/O consanguineous marriage	32	6	38	54.28

Present study shows consanguinity in 38 (54.28%) cases.

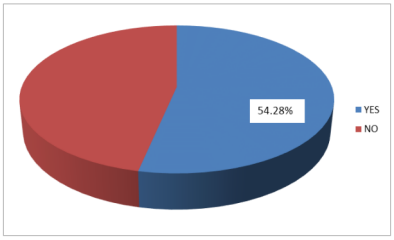


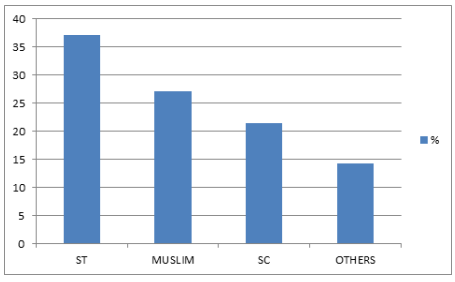
Table no.5: Caste wise distribution of cases in present study:

Caste	Gr-A Sβ thal n=60	Gr-B Eβ thal n=10	Total n=70
Scheduled Tribe (ST)	24	2	26 (37.14%)
Muslim	15	4	19 (27.14%)
Scheduled caste (SC)	13	2	15 (21.42%)
Others	8	2	10 (14.28%)

Sheduled Tribal (37.14%) was the most common ethnic background

among all the groups, followed by Muslims (27.14%).

In HbE-β Thalassemia cases, most common ethnic background was Muslims (4 out of 10 cases).



Distribution of cases in various ethnic groups.

Table no.6: Clinical presentation of cases among all the groups

Clinical features	Gr-A;Sβ thal		Gr-B;Eβ thal	
	n=60	%	n=4	%
Pallor	55	91.66	10	100
Jaundice	22	36.66	3	30
VOC	2	3.33	-	-
Abd pain	15	25	2	20
Leg ulcer	1	1.66	1	10

Majority of the cases presented clinically with pallor (91.66%), other clinical features were jaundice, abdominal pain and vaso-occlusive crisis (VOC).

Table no.7: Distribution of cases with splenomegaly

Age group (years)	Gr-A Sβ thal		Gr-B Eβ thal	
		%		%
0-10	8	26.66	2	33.33
11-20	16	53.33	2	33.33
21-30	4	13.33	1	16.66
31-40	2	6.66	1	16.66
Total cases of splenomegaly	30		6	

Out of 70 cases 36 (51.42%) cases presented as splenomegaly. Out of 30 cases of Sβ thalassemia with splenomegaly, 16 (53.33%) cases were in the 2nd decade of life, while in Eβ thalassemia with splenomegaly, 2 (33.33%) cases were in 1st and 2nd decade of life.

Table no. 8:
Age wise distribution of clinical presentations of Group-A

Age group (years)	Gr-A, Sβ thal, n=60				
	Pallor	Jaundice	VOC	Pain Abd	Leg ulcer
0-10	16 (26.66%)	6 (10%)	-	3 (5.66%)	-
11-20	25 (41.66%)	15 (25%)	2 (3.33%)	10 (16.66%)	-
21-30	11 (18.33%)	1 (1.66%)	-	1 (1.66%)	1 (1.66%)
31-40	2 (3.33%)	-	-	1 (1.66%)	-
>40	1 (1.66%)				

VOC= Vaso-Occlusive Crisis.
The patients belonging to the second decade, most commonly presented with pallor (41.66%) followed by jaundice (25%).

Table no.9: Age wise distribution of clinical presentations of Group-B

Age group (years)	Gr-B, Eβ thal, n=10				
	Pallor	Jaundice	VOC	Pain Abd	Leg ulcer
0-10	5 (50%)	-	-	1 (10%)	-
11-20	2 (20%)	1 (10%)	-	1 (10%)	-
21-30	2 (20%)	1 (10%)	-	-	-
31-40	1 (10%)	1 (10%)	-	-	1(10%)

Pallor was the common presenting feature in all the age groups.

One patient in the first decade of life and one patient in second decade of life also had pain in abdomen.

A case in the 4th decade also presented with jaundice and leg ulcer.

Table no.10:
Mean hematological parameters in all groups

	Group-A Sβ thal	Group-B Eβthal
Hb (gm. %)	7.66	5.58
RBC count (million/cu.mm)	3.13	2.57
PCV (%)	24.85	19.51
MCV (fl)	70.91	69.46
MCH (pg)	24.75	20.25
MCHC (gm. %)	30.54	27.69
RDW (%)	22.09	27.68

Hemoglobin level is decreased in both groups.

Table no.11:
Average Hemoglobin levels in all groups

	Group-A	Group-B
HbF (%)	21.03	27.34
HbA_0 (%)	8.9	10.4
HbA_2 (%)	4.54	55.95
HbS (%)	64.48	-

HbF and HbA2 mildly raised in Group A. Group B shows high HbA2 and HbF.

Compound heterozygous cases presenting with complications:
1) Avascular necrosis of femur
2) Chronic renal failure
3) Growth retardation

Table no.12: 1) Avascular Necrosis (AVN):
A 21 year old female of Sβ thalassemia presented with unilateral AVN of femur.

	Case.1
Age/ Sex	21/F
Pain at hip joint	Present
Necrosis at hip bone	Unilateral
Hb pattern	Sβ thalassemia

2) Chronic Renal failure:
16 year male admitted in the ward with complaints of breathlessness, puffiness of face since 1 month and oliguria since 2 days. O/E- Facial and pedal edema present.

Investigations:
KFT- Sr. Urea -200 mg/dl
Sr. Creatinine -6.2 mg/dl

Diagnosis: Chronic Renal failure in a case of Sickle Beta thalassemia.

3) A case of Sickle with BETA- thal presenting as Growth retardation:
12 year old male with growth retardation and repeated history of transfusion every 4 months.

O/E- Patient having growth retardation.
P/A- Huge splenomegaly Late sickling- Positive

HPLC- S-63.6 % F-21.5%
A2-4.9% A_0-8.1%

Considering clinic-hematological feature (H/O blood transfusion within last 6 months was ruled out) and HPLC- Possibility of Sickle Cell disease with BETA thalassemia. This needs confirmation by genetic study. So, case was referred for further work up. Parental study was not possible in this case.

SUMMARY AND CONCLUSION :
In the present study entitled "Study of double heterozygosity by High Performance Liquid Chromatography (HPLC) in hemoglobin

disorders" total 70 cases of compound heterozygous states were evaluated in the Department of Pathology of Rajendra Institute of Medical Science, Ranchi from July 2013 to August 2014.

1) The compound heterozygous states are grouped as:
   Group A- Sβ thalassemia - 22+38=60 cases (85.71%)
   Group B- Eβ thalassemia - 6+4=10 cases (14.28%)
2) Maximum cases of Sβ thalassemia presented in the late 1st and the 2nd decade. This may reflect that Sβ thalassemia cases remain asymptomatic for comparatively longer period of time than thalassemia and other hemoglobinopathies.
3) 54.28% cases showed consanguinity. This highlights the role of premarital counseling.
4) Scheduled Tribe (ST)- (37.14%) and Muslim (27.14%) were the most commonly affected ethnic groups, this emphasizes on the need to formulate community based studies and implement on the strategies accordingly.
5) Pallor was the most common feature among all the groups followed by jaundice and splenomegaly. Anemia, jaundice and splenomegaly should be investigated in detail for the possibility of Hb disorder.
6) Cases should be investigated by specific algorithm. Even though advanced laboratory methods like HPLC, are indispensable tools in the diagnosis of Hb disorders, they should never be considered the test of first choice in the work up.
7) In asymptomatic cases, Sβ thalassemia cases may be detected for the first time. In such cases the study of spouse for evidence of variant any Hb by HPLC is essential to prevent affected progeny.
8) The cases presenting as complications like growth retardation, aplastic crisis, meningitis etc. with anemia should be investigated by a specific algorithm to rule out possibility of Hb disorders so as to prevent unnecessary medications and surgical interventions.
9) Role of family studies: Family studies are specifically useful in definitively distinguishing homozygous sickle cell anemia from double heterozygous state.
   In the Indian set up where there is paucity of funds and inadequate facilities, simple family study performed in each case serve this purpose. It is a robust and equally efficacious tool as compared to genetic study.
10) In the present study β^0S and β^+S could not be distinguished on the basis of various hemoglobin levels. Genetic study is required for the same.
11) Present study also emphasizes the need of community based targeted study and field work. So that, health care resources can be planned accordingly to reduce the disease burden.

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