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A Study of use of High Performance Liquid Chromatography (HPLC) for diagnosis of Thalassemia

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Abstract: Hemoglobinopathies represents a group of disorders with altered gene mutations running in families that results in variety of disorders. This makes a large number of pools of patients in general population. Many a time's traits of various hemoglobinopathies go undetected and thus increases morbidity. If we could detect these conditions earlier then we can limit morbidity and spread in general population through family extension giving a better life for patients. Thus, the present study carried out to use High Performance Liquid Chromatography in diagnosis of thalassemia and screening of family members of affected individuals with aim to study clinical features, hematological parameters and HPLC findings in patients of Thalassemia. This study included total 127 cases of thalassemia and divided into five groups as thalassemia major- group A, thalassemia minor-group B, Sß$^{+}$ - group C, Sß^{0} - group D and group rare included thalassemia intermedia, delta beta thalassemia, double heterozygous for S and E, etc. maximum number of cases were seen in the age group of 0-10 years in thalassemia major, and 21-30 years in thalassemia trait. Buddhists was common religion having affected individuals. In clinical presentation pallor was common symptom, splenomegaly was seen in Sß^{0}, hepatomegaly in thalassemia major. All hematological parameters were reduced in hemoglobinopathies as compared to control group. Thus to conclude HPLC is useful method for diagnosis of thalassemia

Keywords: Diagnosis, High Performance Liquid Chromatography, Thalassemia.

Introduction: With approximately 7% of the worldwide population being carriers, hemoglobinopathies are the most common monogenic diseases. They were originally found mainly in the Mediterranean area and large parts of Asia and Africa.[1] Almost 25 million people in India are carriers of the beta thalassemia gene with a mean prevalence of 3.3% and 6000 to 8000 children are born every year with thalassemia major.[2] Beta thalassemia syndromes are group of hereditary blood disorders characterized by reduced or absent beta globin chain synthesis mostly inherited as recessive traits.[3] The β thalassemias and their interaction with structural haemoglobin (Hb) variants like HbS and HbE are a major public health problem in India.[4]

Automated cation-exchange high performance liquid chromatography (HPLC) has emerged as an excellent screening and diagnostic tool for beta thalassemia syndromes.[5] HPLC has the advantage of quantifying HbF and HbA_2 along with other haemoglobin variants, screening in a single, highly reproducible system. 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 a clinical laboratory.[6] Study of patients along with family studies by HPLC is very efficacious and cost effective tool for Thalassemia syndromes. This study is carried out to correctly diagnose the patients of Thalassemia by performing HPLC and family studies if possible, and to generate data useful for health care resources. Carrier state identification would be useful to create awareness and to offer options like partner selection, prenatal diagnosis and contraception.

Aims and Objectives

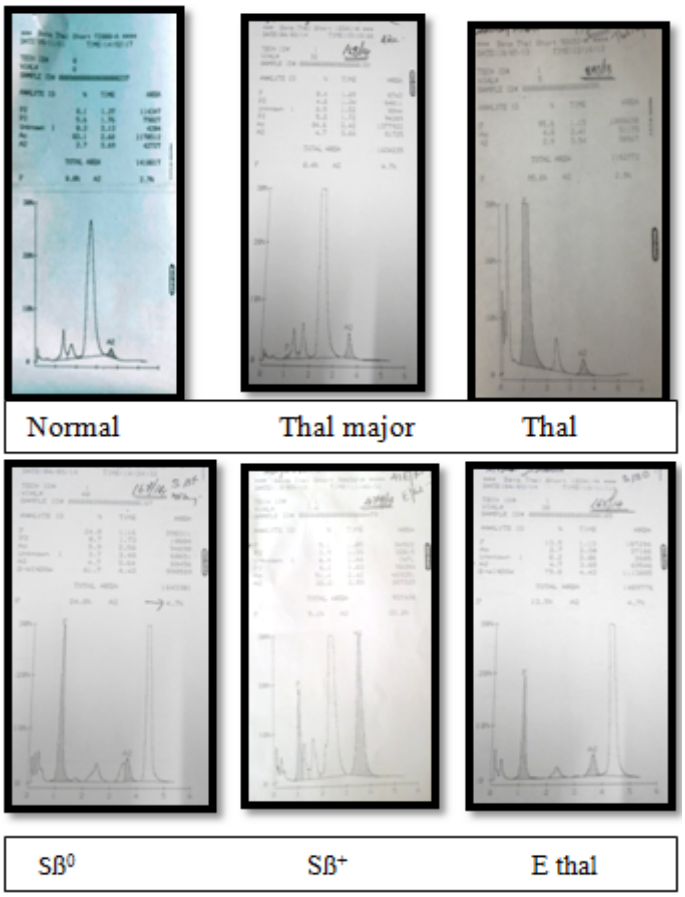
1. To study clinical features, hematological parameters and HPLC findings in patients of Thalassemia.
2. To evaluate the role of family study in patients of Thalassemia.

Material and Methods: The present study carried over two years included patients who are diagnosed as thalassemia by clinical finding, hematological workup, and their family members. After detailed history, complete clinical examination and consent, 2 ml of the patient's blood was collected in 4% K2 EDTA (Ethylene diamine tetra-acetic acid) anticoagulant bulb.

A Specific Algorithm Is Followed In The Study

1. Haematological profile of cases was done. It included Complete blood count using automated analyzer, Peripheral Smear, Reticulocyte count, Electrophoresis (Haemoglobin electrophoresis at pH 8.4-8.6 using cellulose acetate membrane)
2. With the help of HPLC, exact percentage of HbF, HbA2, HbA0, was estimated to diagnose and classify the cases.
3. Then, study of family members whenever possible, was done to confirm the diagnosis and to determine ethnic background and consanguinity.

Automated High Performance Liquid Chromatography (HPLC): Beta thalassemia short program was used in the study in which specific elution windows are defined for abnormal haemoglobins like HbS, HbD, HbE.



Observation and Results: In the present study total 127 cases of Thalassemia were studied.

Table No 1: Cases Were Grouped As:

Group	Gr-A	Gr-B	Gr-C	Gr-D	Gr-Rare							TOTAL
	Thal Major	Thal Trait	Sβ+	Sβ0	TI	δβ	SE	AE	AD	Dβ	Eβ	
No. of Cases	20	59+5*	2+2*	18+4*	2+1*	1	1*	2+2*	3+1*	2	1+1*	127
Total	20	64	4	22	3	1	1	4	4	2	2	
Percentage	15.75%	50.39%	3.15%	17.32%	2.36%	0.79%	0.79%	3.15%	3.15%	1.57%	1.57%	

NOTE: Cases marked by (*) were provisionally diagnosed without family study. Cases with (HbA_2 > 3.9%)

were provisionally diagnosed as thalassemia trait In 110 (86.61%) cases family study was performed.

In 17(13.38%) cases family study was not possible due to various reasons.

As per table no 2 maximum cases were in 0-10 years of age in thal major patients and 21-30 years of age in thal minor patients because this group was mostly comprised of parents of an affected child.

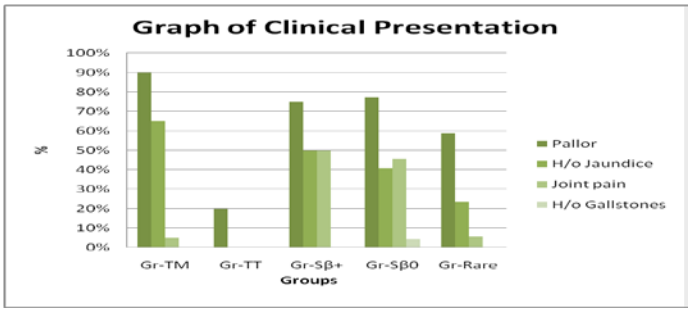
Overall there were 70 males and 57 females in the present study. Present study shows consanguinity in 44 (34.65 %) of cases (gr TM-45%, gr TT-48.44%, Gr SB+-25%, Gr Rare-17.65%) Maximum cases of consanguinity were seen in Muslims 13(29.54%).

Table No 2: Caste wise distribution of cases

		Gr-A	Gr-B	Gr-C	Gr-D	Gr-Rare							
		Thal major	Thal Trait	$S\beta^+$	$S\beta^0$	TI	δβ	SE	AE	AD	Dβ	Eβ	TOTAL= 127	PERCENTAGE
	No. of Cases	20	64	4	22	3	1	1	4	4	2	2		
CASTE	BADHAI	1	2										3	2.36%
	BOUDDHA(SC)	3	11	2	9	1				3	2		31	24.41%
	BRAHMIN		1										1	0.79%
	DHANGAR (NT)		2	1									3	2.36%
	GOND(ST)			1	4								5	3.94%
	GOWARI	1	2										3	2.36%
	GUJRATI	1	2										3	2.36%
	HALBA(ST)	1	4		1								6	4.72%
	KALAR(OBC)				1				2				3	2.36%
	MUSLIM	2	9		4		1						16	12.60%
	KOSHTI(ST)		2		1	1						1	5	3.94%
	KUNBI (OBC)	4	9										13	10.24%
	MALI(OBC)	1	2										3	2.36%
	MANG(SC)				1								1	0.79%
	MARATHA	1	2										3	2.36%
	MEHTAR (SC)	1	2										3	2.36%
	NEWAR(OBC)	1	1										2	1.57%
	PATEL	1	1										2	1.57%
	SINDHI	1	2							1			4	3.15%
	TELI (OBC)	1	10		1	1		1	2			1	17	13.39%

Bouddha (SC**)** (24.41 %) was the most common ethnic background among all groups followed by Teli (13.39%).

Figure 1: Graph of Clinical Presentation



Majority of cases presented clinically as Pallor in all the groups, followed by Jaundice and Joint Pain.

Table No 2: Age distribution (years) in the

	Gr-A		Gr-B		Gr-C		Gr-D		Gr-Rare													
	Thal Major		Thal Trait		$S\beta^+$		$S\beta^0$		TI		δβ		SE		AE		AD		Dβ		Eβ	
Total Cases	**20**		**64**		**4**		**22**		**3**		**1**		**1**		**4**		**4**		**2**		**2**	
Age	n	%	n	%	n	%	n	%	n	%	n	%	N	%	n	%	n	%	n	%	n	%
0 - 10	15	75%	5	7.81%	1	25%	5	22.73%	3	100%	1	100%			3	75%	1	50%			2	100%
11 - 20	5	25%	2	3.13%			9	40.91%									2	100%				
21 - 30			34	53.13%	3	75%	6	27.27%									1	50%	1	50%		
31 - 40			17	26.56%			2	9.09%					1	100%	1	25%			1	50%		
41 - 50			5	7.81%																		
51 - 60			1	1.56%																		

Out of 127 total cases 46(35.66%) cases had splenomegaly, and 18 cases (13.95 %) presented with hepatomegaly. Only 8 cases (6.30%) presented with hemolytic facies.

Table no 4: Mean hematological parameters

	Gr-A	Gr-B	Gr-C	Gr-D	Gr-Rare						
Parameter	Thal Major	Thal Trait	$S\beta^+$	$S\beta^0$	TI	δβ	SE	AE	AD	Dβ	Eβ
Hb(gm%)	6.96±1.85	10.43±1.49	8.55±3.81	7.61±2.4	6.7±0.43	5.9	10.5	10.45±0.46	10.52±1.28	9.15±3.04	6.3±0.42
RBC count (million/cu.mm)	3.65±1.24	5.05±0.89	4.57±1.65	3.68±0.99	3.1±0.45	2.12	4.51	4.6±0.99	4.02±0.65	4.85±1.15	3.27±0.72
PCV (%)	21.06±7.29	33.6±7.89	27.67±12.74	22.92±7.35	20.53±2.46	16.5	30.8	36.17±6.7	31.82±3.84	26.75±9.68	17.95±3.04
MCV (fl)	72.46±7.86	71.27±13.27	74.42±7.83	70.08±11.8	70.03±5.13	78.1	68.5	80.32±14.89	77.42±6.12	54.35±7	55.35±3.04
MCH (pg)	22.13±4.64	21.15±3.67	25.03±8.89	22.34±5.04	22.47±0.92	27.8	23.2	23.57±5.71	27.17±1.07	18.65±1.76	19.85±5.72
MCHC (gm %)	29.39±4.99	27.1±5.89	28.87±5.85	31.35±5.13	31.96±2.45	35.7	34	29.45±4.18	32.97±1.49	34.45±1.2	35.75±8.41
RDW (%)	18.61±3.52	15.49±2.61	18.72±2.5	19.75±3.95	18.73±3.37	30.3	14.3	17.57±1.57	13.78±0.25	18.25±2.05	22.95±4.45
Reticulocyte	6.39±2.24	3.03±0.719	4.35±1.61	5.36±1.91	5.7±0.26	7.5	4.4	2.85±0.44	3.22±0.48	5±2.12	7.5

Thal major patients had low hemoglobin levels (6.96±1.85) as compared to the other groups. In Thal trait group the hemoglobin levels were above 10gm%. RDW was found to be high in Thal major patients and it was (18.61±3.52). Reticulocytes are increased in Thal Major Patients (6.39±2.24) while slight increase was observed in Thal Trait (3.03±0.71)

Table 5 : Peripheral smear findings

		Gr-A	Gr-B	Gr-C	Gr-D	Gr-Rare					
		Thal major	Thal Trait	$S\beta^+$	$S\beta^0$	TI	δβ	SE	AE	AD	Dβ	Eβ
	No. of Cases	20	64	4	22	3	1	1	4	4	2	2
1	Microcytic Hypochromic with Anisopoikilocytosis	5	11	2	10	3			1	2	1	
2	Microcytic Hypochromic with mild Anisopoikilocytosis, target cells	3	2									
3	Microcytic Hypochromic with target cells, Polychromatophilic cells	5	1				1				1	2
4	Normocytic normochromic	3	50						3	2		
5	Microcytic Hypochromic with mild Anisopoikilocytosis, target cells and Basophilic stippling	4										
6	Microcytic Hypochromic with mild Anisopoikilocytosis sickle cells and polychromatophils			2	12			1				

Normocytic normochromic anemia was commonly seen in this study which was common in group B thal trait patents.

Table 6 : Average hemoglobin levels by HPLC

	Gr-A	Gr-B	Gr-C	Gr-D	Gr-Rare						
	Thal Major	Thal Trait	$S\beta^+$	$S\beta^0$	TI	δβ	SE	AE	AD	Dβ	Eβ
HbF (%)	54.52±43.58	1.57±2.39	20.7±12.17	16.64±7.91	28.67±24.15	53.1	3.2	0.93±0.51	0.9±0.08	1.3	13.4±6.08
HbA0 (%)	38.17±37.15	83.11±3.44	10.45±6.14	3.4±1.12	49.2±4.51	42.6	4.5	64.62±1.91	51.92±1.94	5±0.98	29.6±30.8
HbA2 (%)	3.76±1.35	5.28±0.92	6±1.6	6.08±1.18	4.53±1.7	2.9	31.9	26.93±3.91	2.2±0.48	4.05±0.21	54.25±31.18
HbS (%)	-	0.6	61.65±4.9	72.25±6.91	20±20.36	1.6	59.6	-	36.8±1.9	-	-
HbE(%)[E+A2]	-	-	-	-	-	-	-	-	-	-	54.25±17.74

In Thal Major Group the average HbF levels were high as compared to the other groups .The cut off value of HbA2 >3.9%, was used to diagnose Thalassemia trait,7,8 In Thal trait patients the average HbA2 level was found to be (5.57±0.60).

Discussion

Thalassemia trait constituted the commonest disorder with 64 cases (50.39 %) in the present study. Similar results were seen in studies done by Ambekar S.S et al9, Patel J et al10 , Sachdev R et al 11 ,Rao S et al12,C.Vani and S.Mamta13also. There was a high detection of Sickle – Beta Thalassemia (20.47%) cases as compared to the other studies. Tyagi et al14 reported 14.2 years as an average age in Sβ Thal patients. Patel J et al10 reported 90.65 % of Thalassemia major patients belonged to <10 years and 9.38 % were of >11 years of age group C.Vani and S.Mamta13 reported that in their study 16.5% patients were of <12 years of age. In the Present Study out of 127, 70 were males and 57 females which was comparable with the study of Patel J et al48. Bouddha (SC) (24.41%) was commonest ethnic group affected followed by teli (OBC) (13.39%) which was similar to study by Ambekar SS et al9(56%) while Shah SJ et al15 observed that hindu was common region affected followed by muslim. Baig et al16observed that very high (>81%) consanguinity was the risk factor for high incidence of β-thalassemia. J Sana et al 17 (2008) found consanguinity rate of 24.3%. In the present study 34.65% cases were associated with

consanguineous marriage with the maximum cases in Muslim (29.54%).

Tyagi S et al18 in their study on Thalassemia Intermedia observed 71 cases (91%) with splenomegaly & 86 patients (92.5%) with hepatomegaly. Tyagi et al 14 in their study on sickle cell syndrome, found that pallor (62.5%), jaundice (75%),painful crisis (62.5%) and leg ulceration (25%) were more frequent in sickle cell disease than in double heterozygous Sβ Thal patients. Panigrahi I et al 19 in their study on HbEβ Thal patients found that pallor was the commonest feature presenting in all cases (100%), splenomegaly in 17 cases(74%) and hepatomegaly in 15 cases (65%) which was comparable with the present study. However, only 1 patient of Sβ° group had a history of gallstones. Maximum cases of splenomegaly and hepatomegaly were seen in Thalassemia major group followed by Sβ Thalassemia.

As per the studies by Tyagi et al14,Panigrahi I et al19 ,Jha et al 20,Patel.J et al10 ,Rao.S et al12 ,Zakerinia et al21it is observed that low haemoglobin levels in β-thalassemia major <7g%. In Thalassemia Trait group it was (10.43±1.49g %) in present study which was similar to other comparable studies; Patel J et al10 10.14g %(3.2-14.1) and Rao S et al12 (10.3±2.1g %). Thalassemia Major group- MCV was 72.46±7.86 fl in present study which was similar in study reported by Patel J et al10 66.93(55.7-80)fl and Rao S et al12 (74.9±8.5fl). Thalassemia Trait group- in present study MCV was 71.27±13.27fl; which was similar to other comparable studies; Patel J et al10 63.15(48.1-83.1)fl and Rao S et al12 (68.6±7.4fl). Thalassemia Major group- MCH was 22.13±4.64pg in present study which was similar in study reported by Patel J et al10 20.43(12.4-30.1)pg and Rao S et al12 (23.3±3.7pg).Thalassemia Trait group- in present study MCH 21.15±3.67pg; which was similar to other comparable studies; Patel J et al10 18.75(10.1-27)pg and Rao S et al12 (20.5±2.6pg). Thalassemia Major group- MCHC was 29.39±4.99gm % in present study which was similar in study reported by Patel J et al10 29.93(21.6-38.7)gm % and Rao S et al12 (31.1±3.2gm %). Thalassemia Trait group- in present study MCHC was 27.1±5.89gm %; which was similar to other comparable studies; Patel J et al10 29.92(23.5-35.8)gm % and Rao S et al12 (28.3±1.8gm %).

In the present study the average HbF level in thal major group was variable (54.52±43.58), which was comparable to the others studies by Gonzalez-Redondo JM et al 22,Patel J et al10 ,Rao.S et al12 ,Tantawy AG et al 23, C.Vani and S.Mamta13 . This may be due to, the reason, that the cases had received blood transfusion. The average HbA2 level in thal trait group in the present study was 5.28±0.92 % which matched with HbA2 level in studies by Tan GB et al 35– 4.60 %,Fucharoen S et al24 - 5.5 ± 1.26 %,Patel J et al 10– 6.09%. In double heterozugous for sickle thal group it was found that study the average HbS levels were >60 % and results were in concordance with studies by Ambekar SS39,Rao S.et al 12,C.Vani and S.Mamta13. In all the studies the average HbF levels were high >15% while in the present study it was - Sβ°16.64±7.91, Sβ+20.7±12.17. In the present study Average HbE variant levels (in A2 window) in Eβ thal group was (54.25±17.74) which matched with other studies by Sachdev R et al11,Rao S et al 12,C.Vani et al 13,Jha BM et al 20 ie >40% along with raised HbF levels. In HbDβ Thal group of patients, average HbF levels were approximately above 1% , average HbA2 levels were >3.9% and average HbD levels in present study – 86.15±3.04% which was in concordance with findings of Zakerinia et al21 – 57.1-88.3(77.7)%. In δβ-Thalassemia Trait group the HbF level was high > 10% and HbA2

levels < 3 % in the above studies which was in concordance with the other studies by Rao S et al12.

Conclusion

HPLC is the best diagnostic modality for varied spectrum of hemoglobinopathies by direct identification and quantification of hemoglobin variants as well as a screening tool for identification of carriers. HPLC has a magnitude in family screening, premarital screening and prenatal diagnosis in some cases. Thus the present study emphasis the need of community based targeted study and field work so that health care resources can be planned accordingly to reduce the burden of Thalassemia In India.

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