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Original Article

Pathology Section

Analysis of Haemoglobin Profile in Haemoglobinopathies by High Performance Liquid Chromatography and Capillary Zone Electrophoresis Methods: A Cross-sectional Study

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ABSTRACT

Introduction: Mutation or deletion of one of the globin genes of haemoglobin results in thalassaemias and haemoglobinopathies. Two diagnostic modalities that can be used to study haemoglobin abnormalities are High-Performance Liquid Chromatography (HPLC) and Capillary Zone Electrophoresis (CZE).

Aim: To compare HPLC and CZE for evaluating haemoglobinopathies.

Materials and Methods: The present prospective cross-sectional analytical study was done over a period between 1st December 2018 to 30th May 2020 in department of Pathology (Central laboratory), BLDEDU's Shri BM Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka. The blood samples were taken from 48 adults and children and were examined for routine thalassaemia screening using both the modalities (HPLC and CZE), excluding those patients who had undergone blood transfusion within a span of 12 weeks. All samples were analysed using an automated cell counter (Sysmex XN-1000), HPLC (Biorad D-10), and CZE. Parameters studied included: Complete Blood Count (CBC), Haemoglobin A (HbA), Haemoglobin A2 (HbA2), Foetal Haemoglobin (HbF). Chi-square test for association, comparison of means using the test, ANOVA (Analysis of variance) for comparison between and among groups, and sensitivity and specificity analysis were made.

Results: Out of 48 cases, 24 cases were of thalassaemia trait, nine cases were normal, three cases of β-thalassaemia major, two cases of homozygous/double heterozygous for β-thalassaemia and δβ-thalassaemia, one case of sickle cell anaemia, one case of compound heterozygous sickle cell anaemia and β-thalassaemia and one case of compound heterozygous for HbE/ β-thalassaemia. In the remaining seven cases only HPLC was done. When the HbF value is >16.5%, it merges with HbA1c values in HPLC (Bio-Rad D-10), because of which it is challenging to comment about exact values of HbF with the help of HPLC. So for such cases, CZE helped us to evaluate the exact value of HbF.

Conclusion: Both the methods were fairly useful in analysing haemoglobinopathies. However, CZE is preferred over HPLC method. The advantage of Hb electrophoresis over HPLC is that it gives exact values of HbF, which is essential to differentiate thalassaemia intermedia from thalassaemia major. Also, it exactly evaluates the percentage of HbE, which elutes with HbA in the case of HPLC. CZE is complimentary to HPLC, which helps assess exact values of HbF, HbS, and HbE.

Keywords: Anaemia, Genetic haemoglobin disorders, Thalassaemia

INTRODUCTION

Thalassaemias and other haemoglobinopathies are a group of genetic disorders resulting from point mutation or deletion of one of the globin genes of haemoglobin [1]. It is the world's most common genetically inherited disorder [2]. Accurate quantification of different haemoglobin fractions are required to diagnosis these diseases [3]. Till date 1,200 Hb variants have been detected [4]. According to World Health Organisation (WHO) around 5% of world's population are carriers for genetic haemoglobin disorders [2]. Approximately, 1,00,000 thalassaemia major cases are born every year worldwide [5].

Out of the total world's thalassaemia newborns, 10% babies are born in India every year which is equivalent to around 10,000 new cases [3]. In India, frequency of β-thalassaemia in general population is 3.5% to 15% [6]. On an average, India is having more than 25 million thalassaemia carriers in which β-thalassaemia, HbE/β-thalassaemia and HbD-Punjab forms the major bulk [7]. The prevalence of β-thalassaemia carriers in Karnataka is around 2.16% [8].

According to National Family Health Survey (NFHS III), Iron Deficiency Anaemia (IDC) is being the most common cause of anaemia in paediatric age group (prevalence being around 70%) followed by thalassaemia, due to which it becomes very essential to diagnose these two entities properly so that the child gets appropriate treatment [9]. A total of 10,000 new cases of thalassaemia major every year in India is a huge economic burden for the country as the management is expensive [2]. It costs around one lakh indian rupees for management of a child at around three years of age, which keeps on increasing with increase in age [10]. Screening plays a very important role and hence if it is diagnosed in early pregnancy then the couple can be counselled 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.

This study helps to break the dilemma in suggesting the better diagnostic test in work up of haemoglobinopathies so that we can eliminate the need for multiple tests and thereby, helping the patients economically and providing them with accurate diagnosis.

Study objectives

- To characterise haematalogical profiles of different haemoglobinopathies,
- To compare the test results of Hb variants by both modalities (HPLC and CZE).
- To determine which method is more effective for diagnosing haemoglobinopathies.

MATERIALS AND METHODS

A cross-sectional study was conducted in the Department of Pathology (Central laboratory), BLDEU'S Shri B.M.Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka, between 1st December 2018 to 30th May 2020 to detect haemoglobinopathies, after taking the Ethical Committee clearance (IEC 286/2018) and taking consent from all the patients.

Inclusion criteria:

- MCV <72 fL and MCH <27 pg.
- Clinically suspected cases of haemoglobinopathies or β-thalassaemia (splenomegaly, jaundice, pallor, dark coloured urine, delayed growth, fatigue, bone deformities).
- Relatives of the known case of β-thalassaemia major

Exclusion criteria: Patients who had undergone blood transfusion within a span of 12 weeks.

Data Collection

The study included clinically suspected children and adults for haemoglobinopathies. 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 an 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, an automated cation exchange HPLC instrument used to quantify HbA2, HbF, HbA, and screening haemoglobin variants like HbS, HbD, HbE, and HbC. Sebia 2 flex piercing, CZE, which is also used to determine various haemoglobinopathies. A 2% of sodium metabisulphite was used wherever required to confirm the presence of HbS. Parameters analysed were: red cell parameters: RBC count, Hb, MCV, MCH, Reticulocyte count, HbF, HbA2 and HbA values [Table/Fig-1].

S. No.	Red cell parameter	Reference range
1.	RBC count	3.5-5.5 millions/µL
2.	Hb	
	a. 6 months- 10 yrs	11.1-15.5 gm%
	b. Men	13-17 gm%
	c. Women	12-15 gm%
3.	MCV	81-101 fL
4.	MCH	31.5-34.5 pg
5.	Reticuolcyte count	0.5-2.5%
6.	HbF	<1%
7.	HbA2	2.2-3.5%
8.	HbA	95-98%

[Table/Fig-1]: Reference ranges for red cell parameters.

STATISTICAL ANALYSIS

Data was analysed using Statistical Package for Social Sciences (SPSS) for windows (Version 23, IBM Corp., Armonk, NY) Microsoft excel 2016 (Microsoft Corp., Redmond, Washington).

Mean±SD values were calculated for each variable in different groups. Comparison of means of RBC parameters RBC count, Hb%, MCV, MCH and reticulocyte % among different groups of specific type haemoglobinopthies using Analysis of variance (ANOVA) test Mann-Whitney U-test is used for comparing the results of CZE and HPLC analysers.

RESULTS

A total of 48 cases were included in the study, having cases ranging from six months to 35 years of age [Table/Fig-2]. In this study, CZE proved to be superior to HPLC in evaluating different haemoglobinopathies.

Age	Number
6 months-10 years	9
10-20 years	2
21-30 years	18
31-40 years	12

[Table/Fig-2]: Age distribution of the cases.

Out of the total study participants, there were 24 cases of thalassaemia trait, nine normal, three β-thalassaemia major, two homozygous/double heterozygous for β-thalassaemia and δβ-thalassaemia, one sickle cell disease, one compound heterozygous sickle cell anaemia and β-thalassaemia and one case of compound heterozygous for HbE/β-thalassaemia [Table/Fig-3].

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

[Table/Fig-3]: Case summary.

Mean RBC count of β-thalassaemia trait was more than that of β-thalassaemia major. Mean Hb concentration of β-thalassaemia trait was towards the lower limit than other haemoglobinopathies, which showed a significant decrease in Hb concentration. Mean MCV and MCH were decreased in all cases except for sickle cell anaemia. Except for β-thalassaemia trait and normal cases, all other cases showed an increase in observed reticulocyte count.

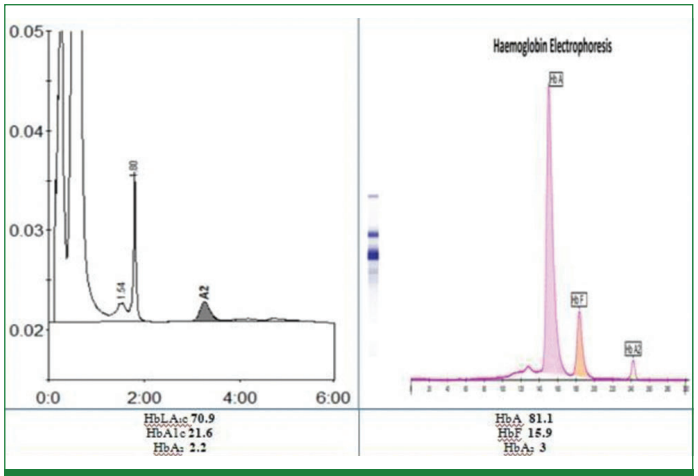
Out of total, 24 cases of β-thalassaemia trait gave the same results in both HPLC and CZE with a very minute difference in the value of HbA2, i.e., HbA2 levels were slightly higher in CZE than that of HPLC. Normal HbF values in an adult is <0.8% and HbA2 is <3.5%. And if HbA2 is between 3.5-3.9%, then it is suspicious of β-thalassaemia trait, while HbF is between 4-7% in β-thalassaemia trait cases.

The low MCV of β-thalassaemia carriers can be masked by the larger size of the HbF cells in cases of β-thalassaemia trait. The Hb concentration in β-thalassaemia trait is usually towards the lower limit of the normal range. Those patients remain asymptomatic till adulthood and are diagnosed while doing the screening.

In BIO-RAD, D-10 dual program (extended), whenever the HbF value is >16.5%, it goes and elutes with HbLA1c, because its normal range is between 0.8-16.5. For such cases, CZE was used to get exact values of HbF, which is essential to make out whether it is thalassaemia major or intermedia. In β-thalassaemia major, the value of HbF is between 30-90%, whereas for intermedia is between 10-30%. It is a

heterozygous condition in which there is an increased synthesis of HbF and decreased/absent synthesis of HbA. Two such cases were diagnosed; both showed raised HbF during early infancy, which raised the suspicion of δβ–thalassaemia or Hereditary Persistence of Foetal Haemoglobin (HPFH).

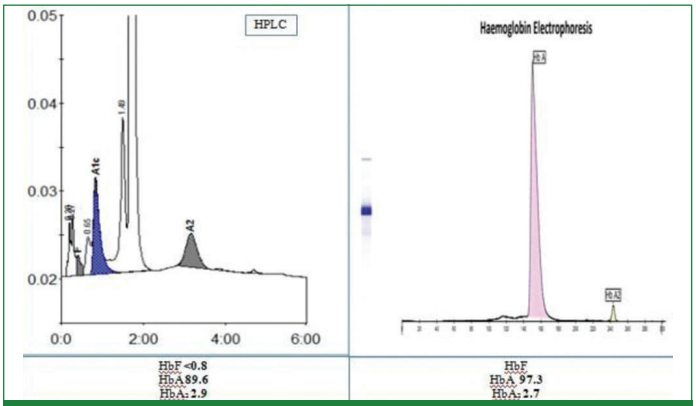
One case of sickle cell anaemia was diagnosed in which the value of HbS was more in CZE compared to HPLC, whereas the value of HbA2 detected was more in HPLC. In this case, the exact value of HbF was not detected in HPLC because it got eluted with HbLA1c. The laboratory investigations values of all cases have been showed in [Table/Fig-4]. The graphical comparison between HPLC and eletrophoresis with respect to valyes of HbF, HbA and HbA2 has been shown in [Table/Fig-5-10] for beta-thalassaemia trait, beta-thalassaemia major, homozygous/double heterozygous for beta thalassaemia and delta beta thalassaemia, sickle cell anaemia, compound heterozygous sickle cell anaemia and beta thalassaemia and compound heterozygous for HbE/beta thalassaemia respectively.



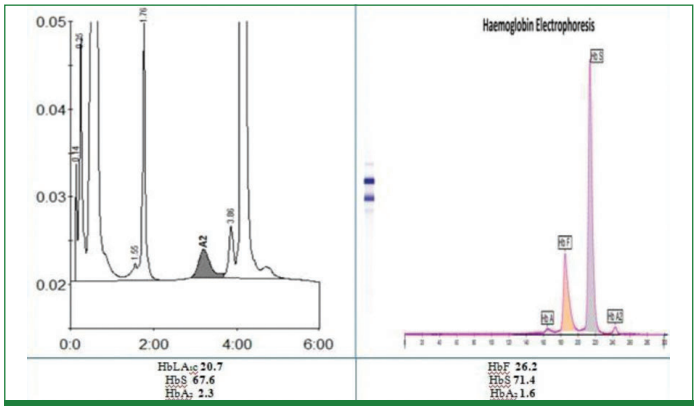
[Table/Fig-7]: Comparision of HPLC and electrophoresis in a case of homozygous /double heterozygous for β-thalassaemia and δβ-thalassaemia.

Variables	n	RBC count		Hb		MCV		MCH		Reticulocyte count	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1. Thalassaemia 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-thalassaemia 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-thalassaemia and δβ-thalassaemia	2	176000.0	9899.5	3.9	0.9	69.4	17.8	21.8	4.0	4.6	4.5
5. Sickle cell anaemia	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 anaemia and β-thalassaemia	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-thalassaemia	1	363000.0	0.0	7.4	0.0	63.1	0.0	20.4	0.0	2.9	0.0
Total	41	48156 1.0	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*	

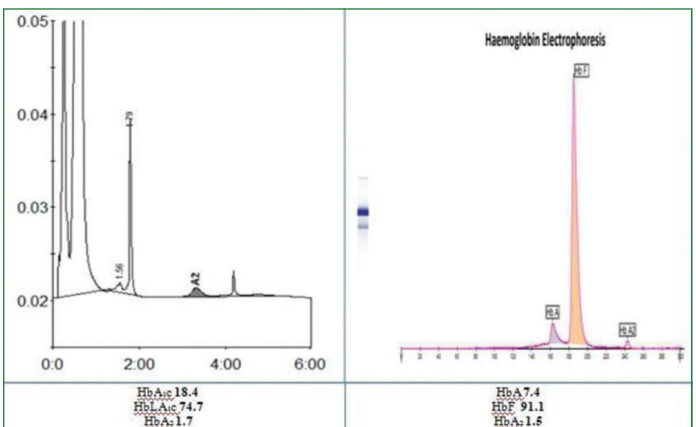
[Table/Fig-4]: Laboratory investigations.
*means Significant (p<0.05)



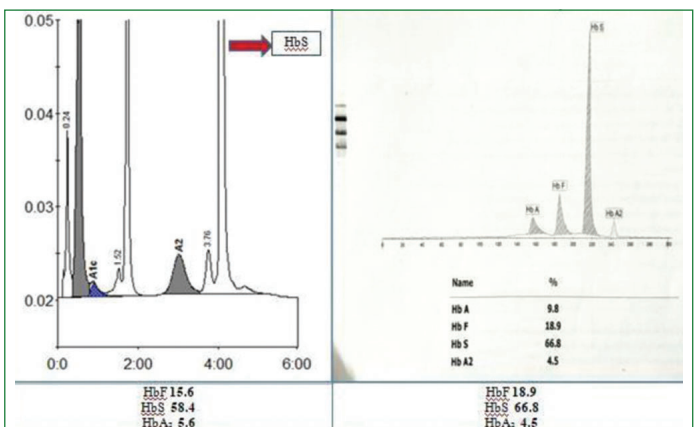
[Table/Fig-5]: Comparision of HPLC and electrophoresis in a case of β-thalassaemia trait.



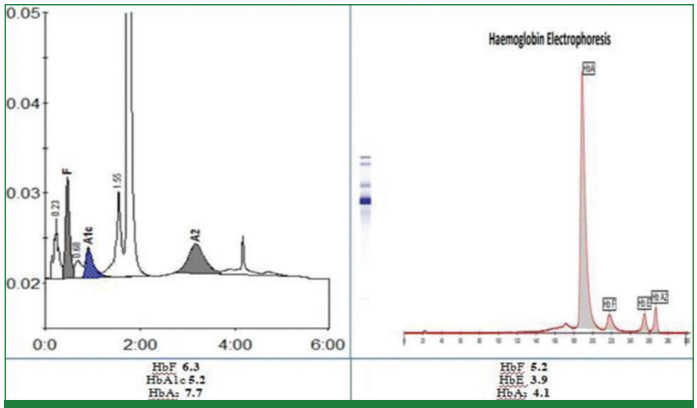
[Table/Fig-8]: Comparison of HPLC and electrophoresis in a case of sickle cell anaemia.



[Table/Fig-6]: Comparision of HPLC and electrophoresis in a case of β-thalassaemia major.



[Table/Fig-9]: Comparison of HPLC and electrophoresis in a case of compound heterozygous sickle cell anaemia and β-thalassaemia.



[Table/Fig-10]: Comparision of HPLC and electrophoresis in a case of compound heterozygous for hbe/β-thalassaemia.

Whether it was sickle cell anaemia or sickle β-thalassaemia, HbS values were high when evaluated by CZE compared to HPLC. Still, both the modalities gave similar data to make a final diagnosis.

Whenever HbA2 values in HPLC are >10%, one should suspect HbD/HbE and go for a complimentary diagnostic modality like CZE, which will precisely detect HbE as observed in this case [Table/Fig-11].

Variables		HPLC	CZE	p-value
HbF	Mean±SD	26.07±35.22	30.66±37.71	0.8345
HbA2	Mean±SD	4.19±1.67	4.03±1.64	0.4637
HbA	Mean±SD	81.08±30.84	82.03±30.64	0.8891

[Table/Fig-11]: Mean and SD of different Hb variants obtained by both the modalities i.e., HPLC and electrophoresis.

DISCUSSION

According to WHO, around 5% of the World's population are carriers of genetic haemoglobin disorder [2]. In India, the frequency of β-thalassaemia in the general population is 3.5% to 15%. At the same time, the prevalence of β-thalassaemia carriers in Karnataka is 2.16% [8].

The most common inherited diseases worldwide are haemoglobinopathies. The prevalence of haemoglobinopathies in India is high due to consanguineous marriages. β-thalassaemia is more common in specific communities such as Sindhi, Gujarati, Bengali, and Punjabi in India, with an incidence of 1-17% [2].

Gender predilection: In this study, patients between the age group 20-35 years were parents of known β-thalassaemia major, which includes mostly mothers accompanying the children; hence female predilection was observed.

CBC parameters of patients with haemoglobinopathies: CBC parameters in this study such as Hb, MCV and MCH were well correlated with the CBC parameters in the study conducted by Khera R et al., as mentioned in [Table/Fig-12,13] [3].

Study	Mean RBC count
Present study	481561±15522
Khera R et al., [3] (2015)	518000±27000
Meenakshi M et al., [9] (2017)	452000±12500
Giordano PC et al., [13] (2014)	600000±10000

[Table/Fig-12]: Studies showing increased mean RBC count in β-thalassaemia trait [3,9,13].

Mean Hb concentration of β-thalassaemia trait is towards the lower limit compared to other haemoglobinopathies, which showed a significant decrease in Hb concentration.

It appeared that MCV and MCH were almost similar in β-thalassaemia major and β-thalassaemia trait. The p-value, which was calculated by using the ANOVA test, was significant for both MCV ($p<0.001$) and MCH ($p<0.001$).

Variables	Present study			Khera R et al., [3]		
	Hb	MCV	MCH	Hb	MCV	MCH
Beta thalassaemia 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 thalassaemia 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 anaemia	7.5	87.2	31	6.4	64.2	17.4
HbS/ β-thalassaemia	9.3	64.5	21.6	8.4± 0.4	64.4± 5.4	21.8± 1.8
HbE/ β-thalassaemia	7.4	63.1	20.4	4.9± 1.9	71.7± 5.8	19.6± 1.8

[Table/Fig-13]: Comparison of CBC parameters between present and study done by Khera R et al., [2].

β-thalassaemia trait: In this study, maximum number was of β-thalassaemia trait cases (58.5%) followed by normal (22.3%), β-thalassaemia major (7.2%), Homozygous/double heterozygous for β-thalassaemia, and δβ-thalassaemia (4.8%). And also there is one case each of sickle cell anaemia, compound heterozygous sickle cell anaemia and β-thalassaemia, and compound heterozygous for HbE/β-thalassaemia.

The prevalence of different haemoglobinopathies in a certain population was correlating with many other studies such as, research done by Khera R et al., Dogaru M et al., and Mondal SK et al., [1,3,11]. [Table/Fig-14]

Study	Present study	Dogaru M et al., [1] (2017)	Khera R et al., [3] (2015)	Mondal SK et al., [11] (2012)
BTT	75%	43.8%	56.3%	63%
BTM	9.375%	1.32%	5.45%	5.5%
HDHBD	6.250%	0.99%		

[Table/Fig-14]: Prevalence of different haemoglobinopathies in comparison with other studies.
BTT: β-thalassaemia trait; BTM: β-thalassaemia major; HDHBD: Homozygous/ double heterozygous for β-thalassaemia and δβ-thalassaemia

Variation in different haemoglobinopathies could be due to less sample size and many cases were not fitting into the inclusion criteria. This high incidence of β-thalassaemia trait suggests that proper screening should be done for women of reproductive age to prevent β-thalassaemia major in the next generation.

Verma S et al., concluded that conditions wherever β-thalassaemia trait is co-existing with iron deficiency anaemia, there HbA2 value is usually low, which can be wrongly interpreted as normal either by HPLC or Hb electrophoresis [12]. This study found nine normal cases, out of which in two cases diagnosis was different with both modalities. CZE diagnosed them as suspicious for iron deficiency anaemia, and HPLC labeled them as normal cases.

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 anaemia and β-thalassaemia trait, but among them, Mentzer's index was concluded as the best by Seighali F et al., [13].

In this study there were two cases in which dilemma was there, so Mentzer's index was used for those two cases. In both, the issues Mentzer's index came out to be >13, which means iron deficiency anaemia, whereas HPLC gave them as normal and electrophoresis gave the result as low HbA2. So for those two cases, serum ferritin was suggested to come to the final diagnosis.

β-thalassaemia major: In this study, there were three cases of β-thalassaemia major. In all these cases, HPLC didn't gave an exact value of HbF because the HPLC model Bio-Rad D10 (extended program); a basic range of HbF which it can calculate is 0.8-16.5% beyond which it goes and elutes with $LHbA_1c$. In contrast, CZE gives an exact value of HbF, which helps differentiate between β-thalassaemia major and intermedia.

Sickle cell anaemia: There were two cases of haemoglobinopathies with HbS component where HbA2 values were higher in HPLC as compared to CZE. A study conducted by Keren DF et al., concluded that in haemoglobinopathies having HbS component, the value of HbA2 will be higher in HPLC than CZE because of co-migration of glycated products of HbS with HbA2 [14].

HbS values in such cases were higher with CZE as compared to HPLC da Fonseca SF et al., suggests using a secondary confirmatory test complementary to HPLC for diagnosing HbS/β° thalassaemia [15].

Compound heterozygous for HbE/β- thalassaemia: Chatterjee JB et al., reported the first case of HbE disease in India [16]. According to Olivieri NF et al., the Indian subcontinent has many cases of HbE trait and Compound heterozygous for HbE/β- thalassaemia [17]. In a multicenter study conducted in Dibrugarh (Assam) incidence of the HbE trait was between 41.1-66.7%[20]. β chain mutation (β26 Glu→Lys) causes HbE thalassaemia [2].

HbE co-elutes with HbA2 in HPLC, making it difficult to segregate and quantify HbA2 in such samples, whereas it is not so with CZE. CZE has the potency to separate HbE from HbA2, which is seen in the case of β-thalassaemia/ HbE, which we observed in this study; this finding is also consistent with the research conducted by Keren DF et al., [14].

In all those cases with Hb variants such as HbS, HbE, etc., the HbA2 value was more in HPLC than CZE. Studies favoring this finding are da Fonseca SF et al., and Greene DN et al., [15,18].

For all those infants in which interpretation was difficult, repeat HPLC and electrophoresis after one year of age and parental screening were 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, and is also consistent with the study conducted by Keren DF et al., [14].

Compared with the survey conducted by Watcharee Prasing et al., our survey gave slightly different results in evaluating HbA2 values in cases of $_2$-thalassaemia/ HbE with CZE [19]. In that study, HbA2 was >6%, while in our study, it is <6%. Simultaneously, there was no significant difference in evaluating HbF by both the modalities.

Limitation(s)

1. Relative small sample size;
2. Instrument specific observation: study used specific models of HPLC and CZE. There could be a slight impact on reproducibility of study results when different models were used.
3. Co-existing conditions were not fully analysed.

CONCLUSION(S)

Exact diagnosis of Hb variants is essential for proper management. It was observed that HPLC and CZE are used for quantification of haemoglobinopathies however CZE gives exact values of Hb variants. Quantification of Hb variants was better with CZE except for beta thalassaemia 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 thalassaemia intermedia from thalassaemia major. CZE exactly evaluates percentage of HbE which elutes with HbA2 in case of Compound heterozygous for HbE/β-thalassaemia in HPLC. CZE is complimentary to HPLC, which is very helpful for evaluating exact values of HbF, HbS and HbE. Cases with low HbA2 should be subjected to iron status study to rule out co-existing IDA.

Author contribution: This study was carried out during (Postgraduation of first author Dr. Pallavi Kommoju in the year 2018-2020). The contribution of each of the authors were: 1st author- conceptualisation, developing methodology and data collection; 2nd author- writing original draft, reviewing and editing; 3rd author- reviewing, editing and statistical analysis; 4th author- Visualisation of results and statistical analysis.

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