

Comparative analysis of the role of isoelectric focusing and high-performance liquid chromatography in newborn screening in hemoglobinopathies

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ABSTRACT

Introduction: Hemoglobinopathies are the common genetic disorders and are considered as the emerging health burden. To reduce the childhood and infant mortality and morbidity, early recognition by newborn screening and timely intervention is necessary. **Objectives:** The objectives of this study are to evaluate the prevalence of different hemoglobinopathies in the study population and to compare the efficacy of isoelectric focusing (IEF) and high-performance liquid chromatography (HPLC) tests in the neonatal screening for hemoglobinopathies. We have also assessed the predominant mutations for all β -thalassemia variants. **Materials and Methods:** The prospective observational study was conducted in the Department of Pathology in collaboration with the Department of Neonatology over 1½ years including 4200 neonates. Both IEF and HPLC diagnoses were attempted and compared with recall HPLC and parent HPLC. DNA analysis was also done further confirmation in all thalassemia cases. **Results:** A total of 213 cases with 11 Hb variants were detected; among them, HbE trait was the most prevalent type. Overall sensitivity, specificity, and negative and positive predictive values were noted. The results of IEF were comparable with HPLC with a statistically significant measure of agreement of $\kappa=0.928$ between the two. DNA analysis of 37 β -thalassemia variants revealed three common mutations, i.e., cd26 (G>A), IVS1-5 (G>C), and cd15 (G>A). **Conclusion:** In IEF, the hemoglobin separation is very precise with little band overlap, but the process and interpretation need a high expertise. Due to more sensitivity, IEF should be the initial screening test followed by recall HPLC for confirmation of the diagnosis.

Key words: Hemoglobinopathies, High-performance liquid chromatography, Isoelectric focusing

Hemoglobin (Hb) disorders, which include thalassemia and Hb variants, are the most frequent clinically significant single gene disorders affecting 7% of the world population [1]. Although sickle cell disease and thalassemia originally restricted to tropics and subtropics, but in this era due to migration, worldwide distribution is noted. To avoid the large financial burden to provide optimal treatment to patients and the innumerable fatalities of untreated patients, screening is the best cure for Hb disorders. Prenatal screening, prospective antenatal screening, and community carrier screening were introduced according to the World Health Organization (WHO) guideline since 1970 [2]. In spite of these, 10,000 newborns are suffering from β -thalassemia every year according to the published epidemiological data in India [3]. The next step of prevention is the secondary prevention which includes early detection by newborn screening to initiate the earliest recognition and timely intervention. Recommended interventions are genetic counseling, disease education, and antibiotic prophylaxis, depending on the diagnosis.

Hb is polypeptide tetramers consisting of two pairs of unlike globin chains (α , β , γ , and δ), to each of which is bound a heme

group. Hb in normal adult human blood is 96% HbA ($\alpha_2\beta_2$), 2–3% HbA2 ($\alpha_2\delta_2$), and 1% HbF ($\alpha_2\gamma_2$). However, the normal newborn blood contains HbF as the major constituent (60–80%) followed by HbA [4]. Hb variants are typically identified from their electrophoretic mobility, the quantity of the abnormal Hb, and the ethnic background of the individual. The electrophoretic methods, still widely used, are declining, given their difficulty of automation and inaccurate quantification of minor Hb constituents. High-performance liquid chromatography (HPLC) is now considered to be a sensitive, specific, and reproducible alternative to electrophoresis. Capillary electrophoresis is also a versatile analytical electrophoresis technique that uses numerous separation principles, including isoelectric focusing (IEF). The majority of Hb variants detected in any laboratory by investigations for hemoglobinopathies are HbE, β thal trait, HbD Punjab, and Hbs [5].

In our study, we aim to evaluate the prevalence of thalassemia and other hemoglobinopathies in the study population and to develop and compare both resolution and quantification capabilities of two methods, suitable for the initial newborn screening: IEF and HPLC assay.

MATERIALS AND METHODS

This prospective and observational study was conducted in the Department of Pathology in collaboration with the Department of Neonatology during January, 2012–July, 2013. Catchment areas were West Bengal and neighboring states (Jharkhand, Bihar, Orissa, and Northeast states) because our hospital was a tertiary care referral center. Prior approval from the institutional ethics committee was obtained. IEF was carried out as primary screening program using dried blood sample by heel puncture on filter papers, followed by HPLC in ethylenediaminetetraacetic acid (EDTA) blood. Based on the initial screening findings, recall HPLC of both infants and parents was further analyzed.

After taking written consent from the parents, dried blood spot samples were obtained from 4200 neonates by heel puncture within 3–7 days of age. Inclusion criteria were (1) non-premature neonates, (2) informed consent from parents, and (3) participants who had no history of blood transfusion at the time of sample collection. The pre-analytical parameters were assessed; complete Hb parameter was measured using 3-ml EDTA blood for study population using automated Hb analyzer ((KX-21, Sysmex Corporation, Japan). Red cell morphology and platelet counts were cross-checked with well-prepared peripheral blood films.

IEF (PerkinElmer, Finland) was employed to detect Hb variant in neonatal screening. RESOLVE® test kits and equipment, together with the IsoScan® Digital Imaging System, was used to detect Hb variants in blood spot samples. PerkinElmer RESOLVE® System consists of Multiphor II Electrophoresis unit, circulating water bath to provide constant temperature control to the electrophoresis unit and programmable power supply. Well number for each specimen was allocated and 3 mm hole, from dried blood spot, was placed in the corresponding well using manual puncher. An amount of 80 µl Hb elution solution was added followed by wait for 30 min at room temperature. The preparation and separation of Hb were accomplished through the application of a hemolysate onto a pre-cast agarose gel, composed of low molecular weight ampholytes with isoelectric points between pH 6 and 8. When electrical current was applied to the gel, these molecules migrate to their isoelectric points (pI) within the gel, forming a stable pH gradient. The Hb variants also migrated through the pH gradient in the gel until the pI of the individual variant equals the corresponding pH of the gel. When the net charges on the variants were zero, the migration ceased. The Hb variants formed a discrete thin band. JB-2 staining system was used for staining of the gel. Results were generated as individual reports for all samples, listing percentage, pI, and comments using the Iso Scan Imaging System.

Hb was reported in decreasing order of concentration. FA, FE, FC, FD, and FAV reporting tables were made and analyzed accordingly. HbA% between 5% and 12% was considered as β -thalassemia trait in non-premature newborns and <5% for thalassemia major and intermedia [6]. Differentiation of major and intermedia was done depending on clinical presentation and pre-analytical red blood cells indices.

In this study, we employed HPLC to validate and tally the results of IEF to find out the positive predictive values between

the 2 exclusive between 3 and 7 days of age. During recall HPLC, 215 abnormal cases and 54 normal cases were found but suspected for parent's history were analyzed. We recruited these data from the age between 60 and 90 days. The data were assembled by the Clinical Data Management software (Bio-Rad Laboratories), and a chromatogram was prepared. Manufacturer-assigned windows for Bio-Rad Variant II HPLC system help in the interpretation of the chromatogram; parent's HPLC was incorporated in abnormal cases.

Molecular analysis at DNA level was done as an additional diagnostic tool in all β -thalassemia variants and α -thalassemia. The DNA was extracted from the blood samples collected from the newborns and their respective parents when they came back to the hospital for recall and follow-ups in 37 cases and 5 cases of α -thalassemia. 1 case of HbS- β -thalassemia and 4 cases of β -thalassemia trait were lost to follow-up. Technicians collected 3 ml of blood from the individuals and further extracted DNA by phenol extraction method using a standard procedure [7]. The DNA-based diagnosis was performed following the methods adopted by Old, 2001 [8], and direct sequencing of hemoglobin beta HBB gene through ABI-3730 DNA analyzer.

The reports of initial IEF and initial HPLC were statistically analyzed based on recall HPLC and parent HPLC using Chi-square test, Matthews correlation test, sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy. Software used in statistical analysis of our study was MedCalc version 11.6 (Mariakerke, Belgium: MedCalc Software 2011).

RESULTS

Among 4200 neonates in this study, we identified a total of 213 cases of hemoglobinopathies consisting of 11 Hb variants (Figs. 1-4) named as HbE, β -Thal Carrier (β +), β -Thal intermediate, HbD Punjab, HbS, β -Thal Major (β 0), HbE/ β , HbD/ β (Hetero), α -thalassemia, sickle β , and HbEE disease. We observed that HbE trait was quite prominent in this region as it was seen in 3.54% within our targeted population and 69.95% among the total Hb variants.

Comparison between IEF and HPLC was also performed. The test group comprised of 215 abnormal cases, which were detected by initial IEF, and control groups comprised of 54 normal cases but suspected on the basis of parent's history. In 269 cases, initial IEF and HPLC diagnoses were attempted and compared with

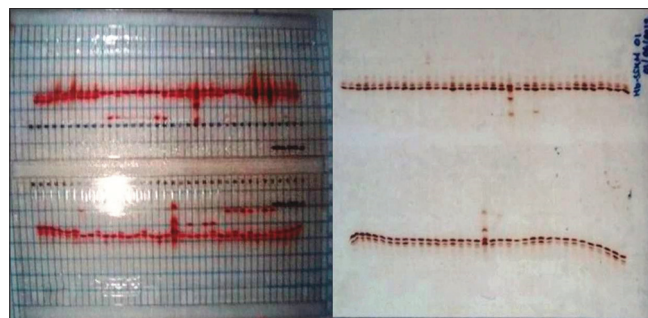


Figure 1: Bar diagram showing major hemoglobinopathies

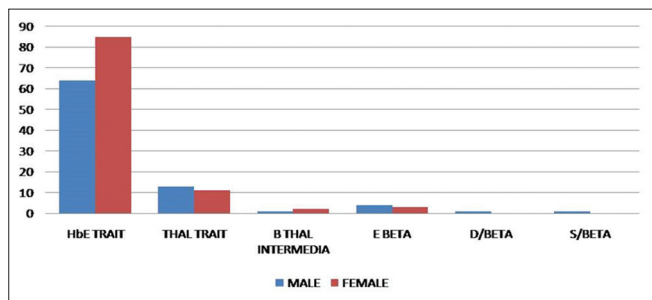


Figure 2: Distribution of other hemoglobinopathies

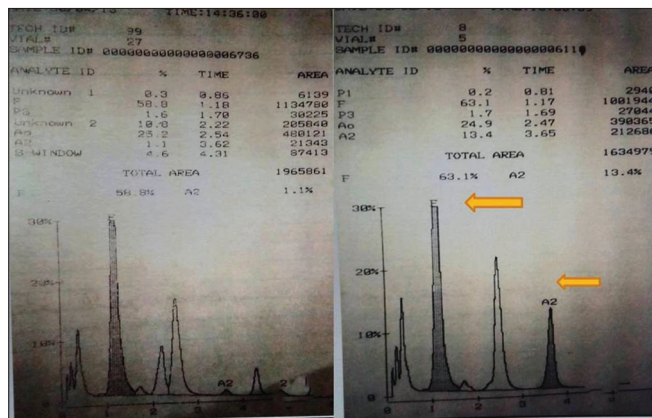


Figure 3: High-performance liquid chromatography curves

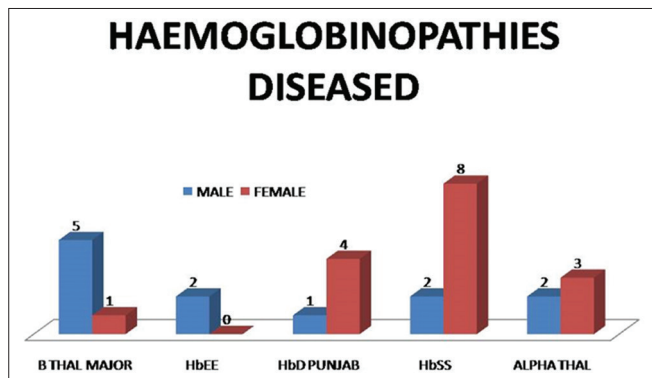


Figure 4: Isoelectric focusing pattern

recall HPLC and parent HPLC. The results are shown in Table 1. Complete correlation between IEF and HPLC was obtained in the diagnosis of thalassemia major (6 cases, 2.81%). There were seven cases of Hb E-β detected by IEF and subsequently confirmed by HPLC. When detecting HbD- β and HbS-β-thalassemia, HPLC was successful in 100% of cases detected by IEF.

Major discrepancies were noted in β-thalassemia intermedia (3 cases, 0.46%). Initial HPLC was able to detect 1 of 3 double heterozygous conditions observed by initial IEF. Chromatograms in two cases were unremarkable. Combination of recall HPLC, parent study, and molecular finding confirmed all three cases of β-thalassemia intermedia. During initial IEF and HPLC, 26 and 24 neonates were suspected, respectively to have β-thalassemia trait depending on the low-intensity band of HbA compared to normal newborns. However, by recall HPLC of suspected cases and parents, only 24 cases were diagnosed and confirmed by further genetic study. A significant drop in the diagnostic accuracy of

HPLC was observed in α-thalassemia. It was identified in 5 cases and 2 cases of the samples tested by IEF and HPLC, respectively. Early eluting peak was detected in HPLC chromatogram in two cases of α-thalassemia. Another 3 cases which were positive in IEF remain undetectable in HPLC probably due to the low concentration of HbH.

No major discrepancies were noted in the initial presumptive diagnosis of other hemoglobinopathies. The most common neonatal hemoglobinopathy in our study was sickle cell disease (10 cases, 4.69%), followed by Hb D-Punjab (5 cases, 2.34%). An abnormality was detected in HPLC chromatogram in all cases of Hb E homozygous (2 cases, 0.93%) detected by IEF revealing significant correlation (100%). The common phenotype in the present study was HbE trait (149 cases, 69.9%). All cases of HbE trait were confirmed to have Hb abnormalities by both HPLC and IEF during neonatal screening.

Evaluation of first line of screening was done, and we documented our experience using IEF and HPLC as the initial newborn screening technique for thalassemia and other hemoglobinopathies. Eleven Hb variants were identified by both the techniques, with no discrepancy in the detection of variants. Overall sensitivity, specificity, and negative and positive predictive values were shown in Table 2. The results of IEF were comparable with HPLC with a statistically significant measure of agreement of $\kappa = 0.928$ between the two.

We have also conducted DNA analysis of 37 β-thalassemia variants for three common mutations found in Asian Indian population, i.e., cd26 (G>A), IVS1-5 (G>C), and cd15 (G>A). The occurrence of these three mutations was seen in a total of 36 cases (87.8%). IVS 1-5 (G>C) was found in 68.3% of cases, cd15 (TGG>TAG) in 21.95% of cases, and cd26 (G>A) was found in 19.5% of cases.

DISCUSSION

Hb disorders are considered to be a serious health problem by the WHO. In India, the carrier frequency of β-thalassemia varies from 1 to 17% (mean 3.3%). In the present study, on 4200 patients, 213 (5.07%) cases were found to have hemoglobinopathies. Although HbE trait was the most common (149 cases, 3.54%) hemoglobinopathies in this study group, it is very closely followed by β-Thal trait (24 cases, 0.57%). There are very few studies from West Bengal which evaluated the prevalence of thalassemia and various hemoglobinopathies. Dolai *et al.* [9] conducted a large HPLC-based study on 35,413 participants from rural Bengal. β-Thal trait was found in 10.38%, HbE carrier in 4.30%, and sickle cell trait in 1.12% of cases. In another study from West Bengal, Jain *et al.* [10] observed that overall 29.3% of subjects were positive for hemoglobinopathies. β-Thal trait was appeared to be the most common hemoglobinopathies in their study followed by HbE heterozygous. In similar study conducted over 3 years in Kolkata on 10,407 participants, 1,509 (14.5%) cases had abnormal Hb fractions. Although β-Thal trait was the most common (579 cases, 5.6%) hemoglobinopathy in this study group, it was very closely (522 cases, 5.0%), followed by HbE carrier [11].

Table 1: Comparison between IEF and HPLC

Hb type	Initial IEF	Initial HPLC	Based on recall HPLC and parent HPLC
Thalassemia			
B-thalassemia major	6	6	6
Hb thalassemia trait	26	24	24
β -thal intermedia/double heterozygous	3	1	3
Hb E/ β	7	7	7
Hb D/ β	1	1	1
Hb S/ β	1	1	1
α -thalassemia	5	2	5
Other hemoglobinopathies			
Hb E trait	149	149	149
Hb SS	10	10	10
Hb D Punjab	5	5	5
Hb EE	2	2	2

IEF: Isoelectric focusing, HPLC: High-performance liquid chromatography, Hb: Hemoglobin

Table 2: Evaluation of IEF as a first-line screening test

Parameters	IEF (%)	HPLC (%)
Sensitivity	100	100
Specificity	99.07	97.65
Positive predictive value	99.95	99.87
Negative predictive value	100	100
p value	<0.0001	<0.0001

IEF: Isoelectric focusing, HPLC: High-performance liquid chromatography

The aim of this was early detection of the Hb disorders to reduce infant morbidity and mortality significantly in the neonatal stage. Early detection helps the clinicians and parents of the affected babies to reach to a decision for early treatment plan such as infants with sickle cell disease must receive prompt follow-up, oral penicillin prophylaxis, and immunizations. Early detection of non-sickle hemoglobinopathies helps in the initiation of prompt delivery of care before the development of clinical complication. Rationality of newborn screening is supported by availability of curative measures like stem cell transplantation nowadays. Parental counseling based on the risk of having next child and education regarding prevention of complication in diseased child is another advantage of the integration of newborn screening. The study confirmed hemoglobinopathies in 213 cases and normal Hb in 3987 cases. The results of IEF were comparable with HPLC with a significant measure of agreement of $\kappa = 0.928$ between the two. We highlight certain advantages of IEF over HPLC in the diagnosis of hemoglobinopathies; the first was excellent Hb separation with very little band overlap.

Normal interpretation of results with HPLC reports in double heterozygous Hb variants was detected due to band overlapping in our study. Some Hbs coelute in the HbA2 window, like HbE, made it impossible to measure HbA2 in the presence of these hemoglobins [12]. In the presence of HbS, HPLC gives falsely increased HbA2 levels, and in HbD, HbA2 is falsely reduced due to an integration

error [13,14]. On the other hand, HbA2 and HbE migrate together in IEF like HPLC, but it is noticeable by their appearance and percentage; hence, we marked a cutoff line to report as follows: If the relative percentage <7% is HbA2 or higher than >10% could be reported as HbE with a clear dark visible band on the gel. Subsequently, distinguish between HbS and HbD Punjab is quite accurate due to their clear view of little distance between the two bands of the variants in IEF. Hempe and Craver [15] showed the excellent role of IEF in neonatal hemoglobinopathies. According to their study, high-resolution separation and quantification of the major and minor Hb variants are possible by IEF. Mario *et al.* [16] stated similar conclusion. Campbell *et al.* [17] demonstrated false negative reporting by HPLC in case of double heterozygous during neonatal screening which is similar to our study.

In our study, we also observed one more advantage of IEF, i.e., accurate detection of α -thalassemia. IEF was more sensitive than HPLC in case of α -thalassemia detection. By protocol, HbH is identified when there are three gene deletions, as HPLC chromatogram demonstrates detectable early eluting peak in > 5% concentration of HbH. In low concentration (<2%), IEF focusing was successful [18]. In spite of the following advantages, we encountered increased figures of false positive report in IEF during detection and evaluation of β -thalassemia traits in the early stage of this study, which was recovered later.

In the present study, DNA analysis has been done on 37 β -thalassemia variant for three common mutations. Among them, IVS1-5 (G>C) is the most common, found in 68.3% of cases, followed by cd15 (G>A) in 21.95% and cd26 (G.A) in 19.5% of cases. Ayub *et al.* [19] conducted a research to see thalassemia mutations in the Bangladeshi population. In this analysis of 587 bp of the HBB gene, splice junction mutation IVS-I-5 was the most common mutation. Gupta *et al.* [20] conducted a DNA analysis in 385 carriers for 5 β -thalassemia mutations. The occurrence of these 5 mutations was seen in 299 (91.2%) carriers. They found Cd16 (-C) in 2.1%, CD30 (G-C) in 1.5%, and CD 15(G-A) in 0.6% which are considered common mutations in the Indian population.

CONCLUSION

The present study revealed that both IEF and HPLC have their own advantages and disadvantages. In IEF, the Hb separation is very precise with a very little band overlap, but the process and interpretation need a high expertise, whereas HPLC is very compact and user-friendly. Although IEF and HPLC both are labor saving and objective screening tool for early detection and management of hemoglobinopathies, IEF should be the initial screening test due to more sensitivity followed by recall HPLC for confirmation and help to take decision regarding treatment

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