Screening of Hemoglobinopathies in 32000 Dried Blood Spot Samples by Cation Exchange High Performance Liquid Chromatography - An Indian Study

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Abstract

Background & objectives:The present study evaluated usefulness of cation exchange highperformance liquid chromatography (HPLC) as a screening tool for early detection of thalassemia & hemoglobin variants in dried blood spot (DBS) samples. We discussed the laboratory aspects ofdiagnostic difficulties in HPLC based screening algorithm in Indian population.Methods:-A total of 32000 DBS samples collected from neonates were primarily screened for hemoglobinopathies on HPLC between June 2013 and May 2016. All presumptive abnormal profiles were re-tested on HPLC after 6 months of age. The follow up results were correlated with parental studies and additional investigations for confirmation.Results: Totally 982 samples (3.06%) showed abnormal HPLC pattern on primary neonatal screening. In follow up testing of 914 presumptive abnormal cases, 583 (1.82%) cases have been confirmed for variant hemoglobin's. Sickle variant was the commonest hemoglobinopathy found in 156 (26.7%) cases. HbE (21.4%), β-thalassemia (19.5%) and HbD (19.5%) disorders being the next common variants. HPLC alone diagnosed more than 85% cases with abnormal variants. Interpretation &Conclusions: Analysis of DBS samples on HPLC is a convenient primary tool for hemoglobin variant screening for hemoglobinopathies. Our study enabled us to design a practical flow chart for large scale HPLC based screening of major hemoglobinopathies with judicious requirement of investigations on other platforms.

Keywords: *High performance liquid chromatography (HPLC), dried blood spot (DBS), hemoglobinopathies*

I. INTRODUCTION

Hemoglobin(Hb) is a tetramer composed of two α-globin and two non-α -globin chains workingin conjunction with heme to transport oxygen in the blood [1].Variant Hb is derived from gene abnormalities affecting the α-globin or β-globin structural genes. More than a thousand Hb variants have been documented, most of which are not clinically relevant [2].However few clinically relevant hemoglobinopathies such as thalassemia and sickle disorders can result in life-threatening symptoms [3].Several Hb variants are prevalent in multiethnic Indian population with an incidence ranging from 3-15%[4,5].

The Maternal and Child Health Bureau of the Health Resources and Services Administration (HRSA) of the United States (US) in conjunction with the American College of Medical Genetics (ACMG) determined few Hb disorders (HbSS, HbS/β-thalassemia and HbSC) to be included them as the core targets of newborn screening (NBS)[6]. The purpose of universal NBS is to identify such cases and provide early education and specialized care prior to the onset of clinical symptoms. Early intervention and potentially curative therapies have significantly improved the prognosis of affected patients [7].Necessity for the neonatal screening in India has therefore been stressed on large scale [4,5].

The laboratory screening of hemoglobinopathies can be achieved by a thorough hematologic evaluation and Hb fraction analysis by high performance liquid chromatography (HPLC). Cation-exchangeHPLChas the advantage of quantifying HbF and HbA2along with other hemoglobin variants in a single run with highreproducibility [8, 9,10]. The simplicityof the HPLC system makesthis an ideal screening methodology for the routine clinical laboratory[11, 12,13].

The present study was conducted to evaluate the role of cation exchange-HPLC along with adjunctive tests for the identification of abnormal Hb variants and screening of clinically relevant hemoglobinopathiesin registerednewborns. Other objectives of the study was to evaluate the model of newborn screening of Hb variants using cord blood and neonatal heel prick DBS sample. The present study also discussed the laboratory perspective of various hemoglobinopathies and diagnostic difficulties in cases with borderline HbA2 values, α-thalassemia and few rare variants which at times require justification on other diagnostic modalities.

II MATERIAL & METHODS

A. Chemicals &Equipment's

This was a retrospective study which included neonatal samples sent to laboratory by clinicians for high-risk and routine screening of hemoglobinopathies.Data comprised ofHb variants analysis of 32000 samples collected between June 2013 and May 2016using modular HPLC system with Empower-3 software (Waters, USA). Demographiccharacteristics, ethnicity, age at diagnosis, gestational age, clinical and the family history of genetic disordersand parental consanguinity were recorded. All reagents and chemicals of analytical grade were purchased from Sigma Aldrich (USA). Automated DBS puncture (DELFIA 1296-071) was purchased from PerkinElmer (USA). Commercial lyophilized control containing mixture of Hb variants were purchased from analytical controls systems, Inc.Cation exchange column and Javelin guard cartridge were purchased from Poly LC Columbia, MD. Deep well plates with pierceablewell cap mat were purchased from Agilent Technologies (USA).

B. Specimen Collection

Umbilical cord blood was collected immediately after birth by the trained hospital staff from umbilical vein with the help of syringe avoiding contamination with maternal blood. Heel prick blood sampleswere collected before 7 days of life as recommended previously [14]. Cord/heel prick blood was taken on preprinted circles of specially manufacturedpaper(Whatman 903).Filter paper sample was allowed to dry properly at room temperature for approximately 2 hours, before being shipped to the laboratory. Written informed consent was obtained from parents of babies. All samples are stored at 2-8^0 C until analysis and analyzed within 48h after its arrival in the laboratory.

C. Laboratory Analysis
1) Sample Preparation

A 3.2 mm diameter disc of collected dried blood specimen was punched into 96 deep well plate using automated DBS puncture. We used buffer A as elution buffer & blood was eluted into0.5 ml elution buffer with 30 min periodic shaking. Pierceable well cap mat was fixed to the plate to avoid sample mixing and also help minimize sample carryover during injection. The plate was centrifuged at 3000 rpm for 15 minutes to settle the filter paper disc at the bottom of the well. Supernatants were transferred to LC auto sampler vials and capped for HPLC analysis. ofsupernatant was injected for analysis.

D. Procedure for HPLC analysis

We used Waters Binary gradient HPLC system with UV visible detector. Auto sampler injections of 10 μL per sample were made into the LC mobile phase flow of 2.00 mL/min. Gradient elution of the analytes was achieved using mobile phase composed of pre-filtered mobile phase A (Tris buffer A pH 6.87) and mobile phase B (Tris-NaCl buffer BpH6.57). Injected sample flows through the column with linear gradient of buffer A and B under constant column temperature of 30° C. The separated Hbfractions were detected through dual-wavelength filter photometer at the characteristic retention time (RT). We used a Cation exchanger column heated at 40 °C, as the primary chromatographic column with guard column. The analysis time per sample was 8 min.

E. Post-analytical Steps:

First, we evaluated the possibility of contamination of cord blood by maternal blood. Cord samples showing >80% of HbA_0 and/or HbA2 >0.5% were suspected for maternal blood contamination and their heel prick/venous sample were processed for re-assessment. Maternal blood contamination was confirmed by comparingHbA_0% in the heel prick and cord blood samples.Samples collected after blood transfusion were mandatorily reanalyzed after 3 months from the last date of transfusion for reliable screening results.

F. Method Validation:

Precision studies were performed using AFSC control sample. Each sample was analyzed in 20 replicates for each HPLC assay for the within-run precision study and analyzed once a day for 10 consecutive days for the between-days precision study. Within run variability of HbA2 and within day variability gave a CV of 0.46% and 3.8% respectively. Limit of detection (LOD) of HbS and HbC for the HPLC method were 0.4% and 0.3% respectively. The method has sensitivity to detect % (ng) of total hemoglobin.

1) Internal Quality Control

Four types of Variant controls i.e. Hb AFSC, Hb AE, Hb AD & elevated A2 were run at the beginning and end of each analytical run or whenever required to lock the RT of each Hb variant. Mean and standard deviation (SD) values for Hb variant concentration were established by replicate analysis of the controls before they were put into use as quality-control action limits. Table 1 showed the variability of controls over 1 year period. Water blanks were included on the calibration tray and on each sample tray for analysis. One retained sample from previous batch was re-analyzed in the next batch to ensure reproducibility. No carryover was observed at any time during the test period during routine sample analysis. Daily analytical processes were handled by personnel trained for the system. Our laboratory participated in the proficiency testing program offered by College of American Pathologists

(CAP) with 100% satisfactory performance for last 5 consecutive years.

Table 1: Internal Quality Control Data of Hb Variant Windows

Hb Variant Peak	Window period	Acceptable range (%)	CV%
Hb F	1.85-2.05	23 - 26	4.81
HbA_0	2.71-3.22	32 – 36	2.94
Hb A2	3.59-3.71	1.3 – 1.9	8.84
Hb S	3.92-4.16	21 - 24	3.13
Hb C	4.52-4.70	14 – 17	3.67
Hb D	3.75-3.95	40 – 45	2.47
HB E	3.38-3.69	27 – 29	2.81

2) *Evaluation of Retention Time (RT)*

At the end of run, the raw data was integrated by the Empower-3 software. The integrated peaks of normal & abnormal controls were assigned to define windows derived from the RT, i.e., the time in minutes from sample injection to the maximum point of the elution peak of Hb variants. The estimation of the fractions is based on the calculation of the areas and valley-to-valley integration mode. The peak eluted at RT not predefined (those mentioned in Table 1) were labeled as an unknown variants. The CV% of the RT normalized to the RT for HbA_0 or the RT for HbA2 for all Hb variants were calculated. There was no statistical difference in imprecision between the mean RT and the mean RT normalized to the RT for HbA_0. For this reason, subsequent analysis of the data was done with only the individual RT. The peak characteristics in addition to the RT were used to identify Hb variants. We used capillary electrophoresis as additional modality for fewcases showing doubtful Hb profile to improve specificity[9].

3) *Chromatographic Data Interpretation*

System generated listing of Hb patterns of all samples in the batch are exported in excel format. The unit of Hb variants were expressed in terms of relative percentage(%) of total Hb.Case with Hb variant having detectable $HbA_0$was considered as heterozygous trait while absence of HbA_0 was considered as homozygous disease. Hereditary persistence of fetal hemoglobin (HPFH) was defined as HbF concentration >10% after 6 months of age. HbH disease was suspected in cases with early eluting (before HbF) one or two peaks >25% during neonatal testing and/orHbA2<2% on follow up testing. Capillary electrophoresis and Parental testing was done for confirmation in doubtful cases[9]. True positives were based on the corroborative results on follow up testing and capillary electrophoresis and, therefore, do not include those cases died or lost during follow up.

4) *Diagnosis of β-Thalassemia*

The significant differences in the Hb variants between different age groups necessitated age related reference ranges of HbA_0, HbA2 and HbF for the diagnosis of thalassemia. At the start of our work, we used reference ranges from the literature [15]-[17].The cut-offs were then refined for different age slots (before 6 months of age) as per our own population-based cutoff values from a large number of normal samples recruited in our screening program as shown in Table 2.

Table 2:Reference Ranges of Study Population (95% Confidence Interval)

Age	Number of cases	Hb F (%)	Hb A (%)	Hb A2 (%)
Cord blood	2000	62-90	14-36	0-0.5
Neonatal period (≤28 days)	2500	28-85	5-72	0-1.5
6-9 months	450	1-5	83-95	1.6-2.5

Cord blood samples with HbF >90%, HbF >85% and Hb A2 >0.5% in first month of life wereretested with heel prick sample in neonatal period. Follow up HbF >5% with variable HbA2 above 3.5% after 6 months of age was considered as confirmation. During routine reporting, we faced problems regarding the reference ranges for thalassemia screening. HbA2 levels may be confounded by several factors [15]. The neonates with presumptive positive results were followed after 6 months of age. The samples with borderline HbA2 (2.5-3.5%) and positive for Hb variants on follow up testingwereretested if it seemed appropriate.Every casewith abnormal Hb profile on follow up testing were correlated clinically with geographical factors, consanguinity, ethnicityand parental study and used Hb electrophoresis to confirm the diagnosis[21].

G Statistical analysis

Continuous variables were expressed as mean ± SD. Categorical variables were presented as frequencies and percentages. All the analyses were done using Microsoft excel 2013.

III. RESULTS

A total of 32000 subjects were screened for hemoglobinopathies during the period of 2 years with male to female ratio of 1.13:1. The age of the study population ranged between 0 days to 11 months with average age of primary screening around 12 days.

We received 10200 cord blood as the first screening sample which constituted almost 32.2% of the total babies screened. About 187 (0.58%) samples have been suspected and then confirmed as contaminated with maternal blood. Among this, 115 cord samples have HbA more than 90% (mean HbA_0 93.4%, range

90.2-97.7%). The distribution of different Hb variants seen in study population was shown in Table 2.

We detected 982 (3.06%) cases with abnormal hemoglobin pattern in the study population in primary screening. 68 cases were lost during follow up. On further testing, 583(1.82%) cases were confirmed for abnormal Hb variants. The distribution of these cases were shown in Figure 1. Among the confirmed positive cases, 46.4% were females and 53.6% were males.
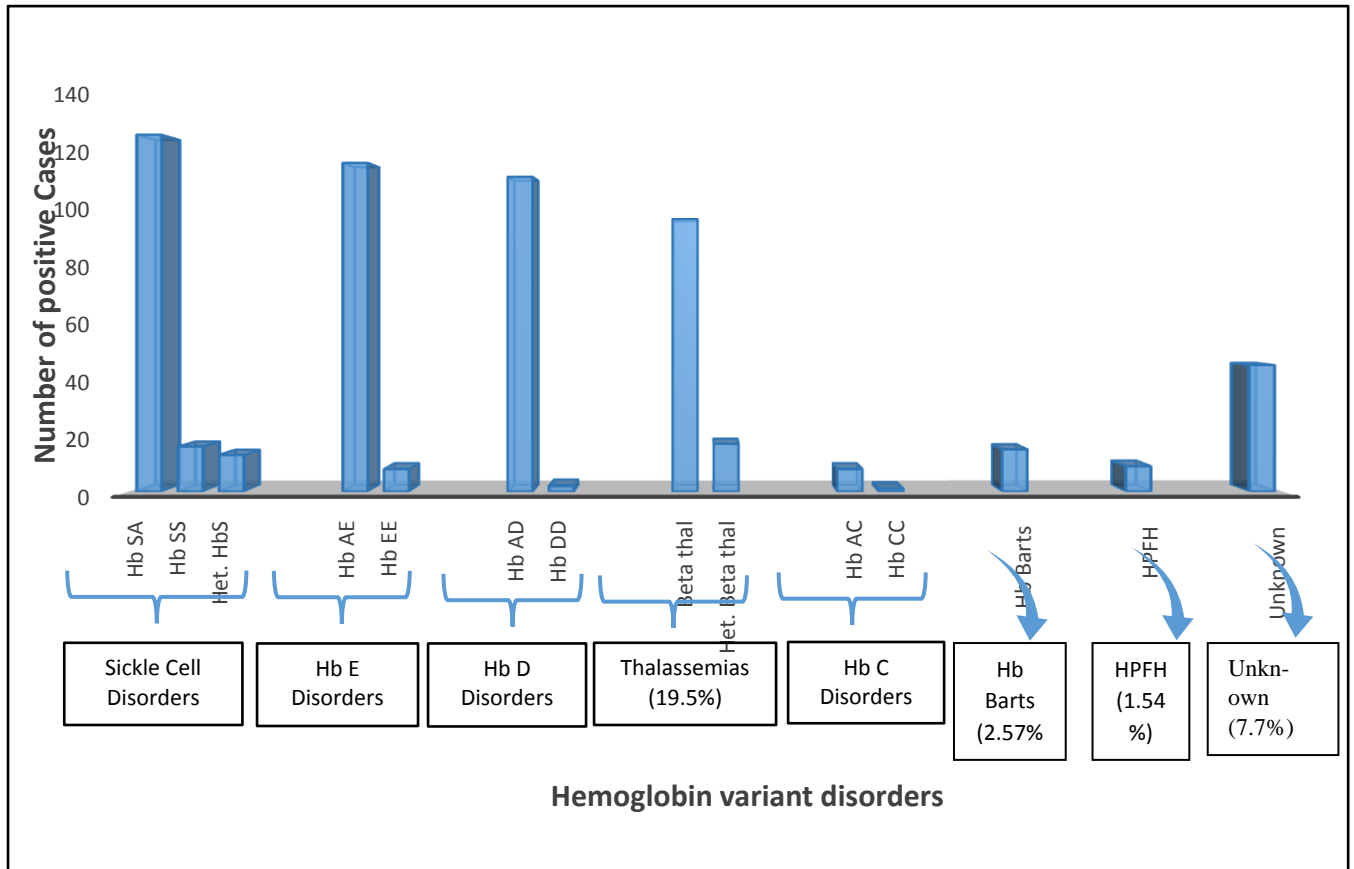

Figure 1: Distribution of Positive Cases of Hemoglobinopathies in the Study Population

Het: heterozygous, HPFH: Hereditary persistence of fetal hemoglobin

The commonest variant in diagnosed case population was sickle cell disorders (27%). Sickle trait was the most common sickle variant. We diagnosed 13 cases with double heterozygotes of sickle disorders (Hb SD and Hb SE). HbE variant disorders were the second common hemoglobinopathies (21.4%) followed by HbD disorders (19.5%).

During initial screening 282 cases of β thalassemia were suspected. After follow up assessment at 6 months, 114(19.5%) cases were confirmed for β-thalassemia. Heterozygous β thalassemia included Hb S/β thalassemia, Hb E/ β thalassemia and Hb D/ β thalassemia.
Uncommon variants found in the study population included Hb Bart's (2.57%), HPFH (1.54%) and Hb C disorders (1.54%).

Apart from the known variants, we detected 41 (7.7%)cases with unknown variants. Non-availability of variant database in our system and loss during the follow up kept their identification beyond the scope of the study.

HPLC established near confirmatory diagnosis inmore than 85%proven cases of hemoglobinopathies with single primary neonatal screening (that included HbS, HbE, HbD, HbC, Hb Bart's and double heterozygotes of sickle and thalassemic disorders). Parental study results were usedonly for confirmation of ambiguous profile especially Hb S/β thalassemia and Hb E/ β thalassemia and few cases of Hb Bart's.

A. Discussion

In Asian subcontinent, India is an ethnically diverse country with marked regional variation whose reflection was seen in the different geographic Hb patterns[5]. Many studies have been published from India on hemoglobinopathies mostly stating the importance of mass screening [18,19]. Such type of situation always demand a neonatal screening method that can detect maximum variants. Ourstudyrepresents the model of neonatal screening of hemoglobinopathies on HPLC system amongst the

routine and high-risk referral cases during the period of 3 years.

The current study was the first ever study in India thatevaluated the practical approach of neonatalscreeningfrom laboratory perspective and discussedprogrammatic issues in hemoglobinopathies screening on DBS samples. The present studyfocused on the development of strategies to streamline the neonatal mass screening and formulate feasible confirmatory algorithm in Indian population.

B. HPLC as Primary Screening Tool

The common tools in the clinical laboratory for identification of Hb variants are column chromatography and electrophoresis [1]. But none of thesemethods can detect multiple common Hb fractions in single run. HPLC has been excellent and powerful diagnostic tool for identification of most of the clinically significant Hb variants in single run owing to its quantitative power and automation [8-10].

HPLC is sensitive, specific, reproducible and required less man power [9].Interpretation of results are less subjective required less expertise, because the peaks are observed in preset windows. In our study, due to small run time, throughput of CE-HPLC increased multifold.Despite allowing adequate time for routine maintenance and running retention markers,one HPLC system can process 120 samples per day and have potential to screen upto36000 samples/year, based on 300 working days in a year. One column can be used for 500 injections. Hence, use of HPLC isacost-effective technology for population screening and routine clinical laboratory with high work load. Other advantages include minimal sample preparation, small sample volume requirement and the ability of the autosampler to analyze four 96 well plates (up to 350 samples)automatically and sequentially.Ease of operation with high throughput makes HPLC system mostsuitable first line screening tool for laboratories with considerable work load.

We have adopted two stage Hb variant testing that is,primary neonatal screening and follow up testing after 6 months of age. Such protocol helped us for presumptive identification of Hb variant as well as introducing a scheme for the reporting of abnormal screening results. Our data showed that we haveunambiguously diagnosed more than 85% positive cases using HPLC system only, that includedHb variants commonly prevalent in Indian population (such as HbS, HbE and HbD etc). Confirmatory testing on other platforms is rarely indicated in majority of such cases. However application of capillary electrophoresis enhances the specificity of HPLC results.

C. Problems with β-Thalassemia Screening

HbF and HbA2 content have an important diagnostic implications in thalassemia screening. Besides β-thalassemia, elevated HbF levels were seen in sickle cell disease, various double heterozygous states such as β-thalassemia trait/HPFH [3].

In our study population, β-thalassemiais one of the largest subgroup of hemoglobinopathies. Iron deficiency may lead a low HbA2 and hence may mask a thalassemia trait, whereas B12/folate deficiency may lead to slightly raised HbA2 leading to a false diagnosis of a trait[15].Conditions with borderline HbA2 therefore were careful interpreted in the light of thorough investigations. According to our experience, β-thalassemia carriers showed ambiguous Hb profile during early screening. HbF >85% has better positive predictivity during early neonatal screening of β-thalassemia than HbA2 >0.5%. We had high percentage of sample recall rate in our screening protocol that was attributed to complexity of the phenotype and ambiguity of HbF and HbA2 levels during neonatal period.

D. Cord Blood Screening

The use of cord blood samples have some advantages against heel prick sample or whole blood in tubes. The quality and stability of the sample is better and the result is available very rapidly after birth. It was concluded that both types of sample were equally reliable for use in neonatal screening especially in clinically relevant hemoglobinopathies such as thalassemia major and sickle disorders [21,22]. So, screening programme, which uses cord blood, enables confirmatory samples to be obtained from children suspected to be affected by hemoglobinopathies before the infants are discharged home. In countries like India, where women are usually discharged one day after delivery, cord blood is the easiest material to collect. Moreover, the nursing staff have found the collection of cord blood as more acceptable to families than heel pricks.

The residualcord sample can be used for confirmatory techniques (e.g. DNA testing). Good sensitivity and superior separating power for quantifying various Hb variants in presence of HbF, the HPLC method is suitable for cord blood screening.

The major challenge of the use of this type of sample is the risk of maternal blood contamination that leads to spurious Hb phenotype. Our experience showed that maternal contamination would need to be very high to alter the neonatal Hb profile and the phenomenon was rarely observed with trained staff (87/10200; 0.85%).

Neonates with abnormal cord blood Hb profile during screening should receive special attention. Those affected by sickle cell disorders can benefit from an early diagnosis and be given comprehensive care. In the case of thalassemia major, neonates will be referred to dedicated health-care staff and the parents will benefit from genetic counselling.

E. Advantages of DBS Analysis

The blood collection on filter paper has several advantages over blood in vacutainers. Filter

paper collection is simple to perform and requires minimal training. The DBS represents a low infectious hazard and risks associated with shipping can beminimized [22]. Blood sample on filter paper is spread based on the hematocrit content. Therefore DBS specimen collectionrequire adequate hematocrit (40-60%) in newborn screening analytes such as phenylalanine, thyroxine etc). However variant Hb screening can be done in samples with varying Hb concentrationsince relative percentage of Hb variants wouldremain unaffected. The HPLC based analysis required only a small sample volume (around 50 μl in one blood spot). Out of which only one punch of 3.2 mm diameter disc (equivalent to 3.1 μl)is required for analysis.Heel prick collection thus preservedlarger veins for parenteral therapy. The early screening protocol enabled us prompt diagnosis of hemoglobinopathies before infants are discharged home, thus offering adequate parental counseling. Early screeningespecially in sickle cell disease directed early intervention with prophylactic penicillin to ameliorate medical complications and reduce morbidity and mortality.

Disadvantages of DBS based analysis included the step of specimen elution into the microplate, which adds to the cost of analysis. Another challenge in the DBS analysis is concentration of the Hb in the hemolysate [23]. This depends on the on the saturation of the filter paper and on the degree of degradation of the Hb. We can the Hb concentration in the hemolysate by prolonging the elution time. However, such drastic elution causes increased chances of cellulose particles being detached from the paper contaminating the hemolysate. Such particle can clog in the system causing high background and spurious results.

F. Challenges

There were fewtechnical limitations in ourstudy. Hemoglobin profiling of cord blood wascomplicated by the presence of relatively high amounts ofHbF especially in preterm babies. The effect of gestational age on the percentage of HbA_0 was evaluated in a subgroup of 2000 neonates for whom the exact gestational age was recorded (data not shown). High HbF necessitated repeat screening on heel prick or venous blood samples after 40 weeks of gestational age and the need for correlation of HbF content with gestational age.

We found significantly higher levels of HbA2 in HbS carriers as compared to the normal individuals. Such observation could be due to the co-elution of glycated sickle hemoglobin with HbA2 [22].Therefore HbA2 cut-off of 5.0 for sickle carriers and 5.5% for sickle cell anemia was proposed.

Since HbLepore and HbE were co-eluted with HbA2, their presence in the sample gave a percentage of HbA2 greater than 10%. Therefore such samples were validated withother modalities for the Hb variant detection.

We reported few cases of δβ-thalassemia and HPFH based on the basis of high HbF. However details of these cases and follow up studies for confirmation could not be done.

Our method is developed in-house and we used few available standards for unknown variants. Therefore we could not identify few cases with variants other than those covered during method validation. The overlap of RT precluded definitive identification of Hb variants by HPLC alone. Such limitation encouraged us to develop the library of hemoglobin variants with the characteristic RTfor identification of some of the rarely observed variants.

The present study reflected only the magnitude of abnormal hemoglobins in a small selected population that may be, in fact the tip of an iceberg. However, our study would definitely help to create awareness about universal screening of clinically relevant hemoglobinopathies among both health care providers and stakeholders in laboratory diagnostics.

IV. CONCLUSION

The present findings showedCE-HPLC as an excellent, powerful diagnostic tool for the direct identification of hemoglobin variants with a high degree of precision in the quantification of normal and abnormal hemoglobin fractions. HPLC based primary neonatal screening model using DBS testing can diagnose important Hb variants most prevalent in India and only a few variants require other platforms for validation. Practicing of proper screening algorithm forHPLC system is cost-effective obviating the need for other modalities.

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REFERENCES

[1] H. Wajcman, K. Moradkhani, Abnormal hemoglobin's: detection & characterization, Indian J Med Res. 134 (2011) 538-546.

[2] B. Hedlund, Hemoglobin's of human embryos, fetuses, and neonates, In: Fairbanks VF, ed. Hemoglobinopathies and thalassemia's, New York: Brian C. Decker, (1980) 14-17.

[3] G.M. Clarke, T.N. Higgins, Laboratory investigation of hemoglobinopathies and thalassemia's: review and update, Clin Chem. 46 (2000) 1284-1290.

[4] T. Chatterjee, A. Chakravarty, S. Chakravarty, Population Screening and Prevention Strategies for Thalassemias and other Hemoglobinopathies of Eastern India, Experience of 18,166 cases, Hemoglobin. 39 (2015) 384-388.

[5] S. Iyer, S. Sakhare, C. Sengupta, A. Velumani, Hemoglobinopathy in India, Clin ChimActa. 444 (2015) 229-233.

[6] D.J. Dietzen, P. Rinaldo, R.J. Whitley, W.J. Rhead, W.H. Hannon, National academy of clinical biochemistry laboratory medicine practice guidelines: follow-up testing for metabolic disease identified by expanded newborn screening using tandem mass spectrometry; executive summary, Clin Chem. 55 (2009) 1615-1626.

[7] B. Modell, M. Darlison, Global epidemiology of hemoglobin disorders and derived service indicators, Bull World Health Organ. 86 (2008) 480-487.

[8] A. Joutovsky, J. Hadzi-Nesic, M.A. Nardi, HPLC retention time as a diagnostic tool for hemoglobin variants and hemoglobinopathies: a study of 60000 samples in a clinical diagnostic laboratory, Clin Chem. 50 (2004) 1736-1747.

[9] D.F. Keren, D. Hedstrom, R. Gulbranson, C.N. Ou, R. Bak, Comparison of SebiaCapillarys capillary electrophoresis with the Primus high-pressure liquid chromatography in the evaluation of hemoglobinopathies, Am J Clin Pathol. 130 (2008) 824-831.

[10] J.W. Eastman, R. Wong, C.L. Liao, D.R. Morales, Automated HPLC screening of newborns for sickle cell anemia and other hemoglobinopathies, Clin Chem. 42 (1996) 704-710.

[11] C.N. Ou, C.L. Rogerud, Rapid analysis of hemoglobin variants by cation-exchange HPLC, Clin Chem. 39 (1993) 820-824.

[12] B.J. Wild, A.D. Stephens, The use of automated HPLC to detect and quantitate haemoglobins, Clin Lab Haematol. 19 (1997) 171-176.

[13] C.N. Ou, C.L. Rogerud, Diagnosis of hemoglobinopathies: electrophoresis vs. HPLC, Clin ChimActa. 313 (2001) 187-194.

[14] J.V. Mei, J.R. Alexander, B.W. Adam, W.H. Hannon, Use of filter paper for the collection and analysis of human whole blood specimens, J Nutr. 131 (2001) 1631S-1636S.

[15] A. Giambona, C. Passarello, M. Vinciguerra, R. Li Muli, P. Teresi, M. Anza, Significance of borderline hemoglobin A2 values in an Italian population with a high prevalence of Beta-thalassemia, Haematologica. 93 (2008) 1380-1384.

[16] F.P. van der Dijs, G.A. van den Berg, J.G. Schermer, F.D. Muskiet, H. Landman, F.A. Muskiet, Clin Chem. 38 (1992) 1864-1869.

[17] A. Mosca, R. Paleari, D. Leone, G. Ivaldi, The relevance of hemoglobin F measurement in the diagnosis of thalassemias and related hemoglobinopathies, Clin Biochem. 18 (2009) 1797-801.

[18] M.K. Baruah, M. Saikia, A. Baruah, Pattern of hemoglobinopathies and thalassemias in upper Assam region of North Eastern India: high performance liquid chromatography studies in 9000 patients, Indian J PatholMicrobiol. 57 (2014) 236-243.

[19] R.S. Balgir, Spectrum of hemoglobinopathies in the state of Orissa, India: a ten years cohort study, J Assoc Physicians India. 53 (2005) 1021-1026.

[20] D.D. Suh, J.S. Kruss, K. Bures, Influence of hemoglobin S adducts on hemoglobin A2 quantification by HPLC, Clin Chem. 42 (1996) 113-114.

[21] P.C. Giordano, Strategies for basic laboratory diagnostics of the hemoglobinopathies in multi-ethnic societies: interpretation of results and pitfalls, Int J Lab Hematol. 35 (2013) 465-479.

[22] S.P. Parker, W.D. Cubitt, The use of the dried blood spot sample in epidemiological studies, J Clin Pathol. 52 (1999) 633-639.

[23] C. Panadea, J.R. Eckman, R.S. Kuchnert, A.F. Platt, Comparison of liquid and dried blood for neonatal hemoglobin screening: Laboratory and programmatic issues, Pediatrics. 93 (1994) 427-432.