

Original Research Article

COMPARATIVE DIAGNOSTIC EVALUATION OF ALKALINE HAEMOGLOBIN ELECTROPHORESIS AND CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN HAEMOGLOBINOPATHIES

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Received : 14/08/2026
Received in revised form : 05/09/2026
Accepted : 17/09/2026

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DOI: 10.70034/ijmedph.2026.3.922

Source of Support: Nil,

Conflict of Interest: None declared

Int J Med Pub Health
2026; 16 (3); 5984-5991

ABSTRACT

Background: Accurate identification and quantification of haemoglobin variants are essential for diagnosing haemoglobinopathies and detecting carriers. Although alkaline agarose-gel electrophoresis is widely available and economical, variants with similar electrophoretic mobility may be misclassified. This study compared cation-exchange high-performance liquid chromatography (CE-HPLC) with alkaline agarose-gel electrophoresis for haemoglobinopathy diagnosis and evaluated their quantitative and operational performance.

Materials and Methods: This prospective comparative study initially evaluated 805 participants referred for haemoglobinopathy testing. Seven participants with recent blood transfusion were excluded. EDTA-anticoagulated blood from the remaining 798 participants was analysed using the Bio-Rad Variant II CE-HPLC system and alkaline agarose-gel electrophoresis at pH 8.6. Haemoglobin phenotypes and HbA, HbA₂, HbF and variant fractions were compared. Intermethod agreement was assessed using Cohen's kappa, while paired differences, correlation coefficients and McNemar's test were calculated as appropriate.

Results: CE-HPLC identified 635 participants (79.6%) with abnormal haemoglobin patterns and 163 (20.4%) with normal patterns. Binary classification agreed in 794/798 cases (99.5%; $\kappa=0.985$, $p<0.001$), with no significant systematic disagreement (McNemar $p=0.125$). Exact phenotype agreement was observed in 790/798 cases (99.0%; $\kappa=0.982$, $p<0.001$). Electrophoresis reported four β -thalassaemia-trait cases as normal, two HbDD cases as HbSS, one HbAD case as HbAS and one Hb Lepore case as HbAE. Among normal participants, mean HbA, HbA₂ and HbF values were significantly lower by CE-HPLC than by electrophoresis (86.90% versus 96.50%, 2.80% versus 3.20%, and 0.78% versus 2.10%, respectively; all $p<0.001$). CE-HPLC provided automated peak integration and completed each analysis in approximately 6.5 minutes, whereas electrophoresis required approximately 30 minutes of migration followed by multiple manual processing stages.

Conclusion: Both methods demonstrated near-perfect diagnostic concordance. CE-HPLC offered faster processing, more objective quantification and better differentiation of variants co-migrating at alkaline pH. Nevertheless, borderline or unusual chromatograms require correlation with haematological findings and confirmation using an orthogonal or molecular method.

Keywords: Alkaline agarose-gel electrophoresis; cation-exchange HPLC; haemoglobin variants; haemoglobinopathies; HbA₂; β -thalassaemia.

INTRODUCTION

Haemoglobinopathies are inherited disorders caused either by structural alteration of globin chains or by reduced globin-chain synthesis, as in the thalassaemias. Their phenotypes range from clinically silent carrier states to severe anaemia, vaso-occlusive complications, transfusion dependence and premature mortality. The Global Burden of Disease Study estimated that 7.74 million people were living with sickle cell disease in 2021, when approximately 515,000 affected births occurred worldwide.^[1] Beta-thalassaemia also remains widely distributed, with the greatest burden in the Mediterranean region, Middle East, South Asia and Southeast Asia.^[2] India has a particularly heterogeneous distribution of beta-thalassaemia, HbS, HbE and HbD-Punjab because of its ethnic diversity, endogamy and regional variation; national guidance therefore emphasises systematic screening, accurate carrier detection and genetic counselling.^[3,4]

Reliable laboratory diagnosis is essential for distinguishing carrier states from clinically significant disease, identifying couples at reproductive risk and avoiding misclassification of microcytic anaemia. Interpretation should integrate clinical history, transfusion status, complete blood count, red-cell morphology and quantitative haemoglobin analysis.^[5,6] Alkaline agarose-gel electrophoresis separates haemoglobins according to electrophoretic mobility and remains relatively economical and accessible. However, visually similar or co-migrating fractions—particularly HbS with HbD/HbG and HbA₂ with HbE/HbC—may restrict variant identification, while densitometric measurement of low-concentration fractions can be operator-dependent.^[7,8]

Cation-exchange high-performance liquid chromatography separates haemoglobin fractions according to ionic interaction and characteristic retention time. Automated analysis offers rapid processing, objective peak integration and precise quantification of HbA₂ and HbF, supporting the detection of beta-thalassaemia trait and several structural variants.^[8-10] Nevertheless, co-elution, unknown peaks, nutritional deficiencies and recent transfusion may complicate interpretation; ambiguous patterns require confirmation by an independent method or molecular testing.^[5,6,9] Direct comparison with conventional electrophoresis is therefore important in resource-variable settings. The present study compared the diagnostic concordance, haemoglobin-fraction quantification and practical performance of cation-exchange HPLC and alkaline agarose-gel electrophoresis among patients evaluated for suspected haemoglobinopathies.

MATERIALS AND METHODS

Study design and setting: This prospective comparative study was conducted in the Department

of Pathology, of a tertiary care centre, in Central India over one year, from June 2012 to May 2015. Each eligible blood sample was evaluated by cation-exchange high-performance liquid chromatography (CE-HPLC) and alkaline agarose-gel haemoglobin electrophoresis.

Study population and eligibility criteria: Paediatric and adult patients referred with suspected haemoglobinopathy were considered eligible. The indications for testing included microcytosis with a mean corpuscular volume ≤ 80 fL, mean corpuscular haemoglobin < 27 pg, sickled erythrocytes on peripheral blood smear, a positive sickling-solubility test, clinical features suggestive of a haemoglobinopathy, or a known family history of an inherited haemoglobin disorder. Family members were investigated whenever clinically indicated and available. Neonates and individuals who had received a blood transfusion during the preceding four months were excluded because transfused erythrocytes could alter the proportions of haemoglobin fractions. A total of 805 individuals were initially recruited; seven were excluded after documentation of recent transfusion, leaving 798 participants for analysis.

Blood collection and preliminary haematological evaluation: Approximately 3 mL of venous blood was collected aseptically into dipotassium ethylenediaminetetraacetic acid tubes. Samples were analysed promptly; when immediate testing was not possible, they were stored at 2–8°C and processed within seven days. Complete blood counts were performed using a Beckman Coulter HMX five-part differential haematology analyser. Peripheral blood smears were prepared simultaneously, stained with Leishman stain and examined for microcytosis, hypochromia, target cells, anisopoikilocytosis, polychromasia, nucleated erythrocytes and sickled cells. A sickling-solubility test was performed in relevant cases. Brilliant cresyl blue staining for haemoglobin H inclusions was undertaken when alpha-thalassaemia or haemoglobin H disease was suspected.

Alkaline agarose-gel haemoglobin electrophoresis: Haemoglobin electrophoresis was performed using the Maestro 101 system (Biotech-Fischer) with agarose gel at alkaline pH. Packed erythrocytes were separated from plasma and buffy coat by centrifugation and washed three times with 0.85% sodium chloride. The washed cells were lysed with double-distilled water to prepare the haemolysate. Approximately 3 μ L of haemolysate was applied to each sample position on the agarose gel. Electrophoretic migration was performed at 150 V for 30 minutes. The gel was subsequently fixed using an ethanol-distilled water-acetic acid mixture, dried, stained with Amido Black, destained with 3% acetic acid and redried. Haemoglobin bands were identified according to their electrophoretic mobility and quantified using a TurboScan densitometer. Samples demonstrating bands in the HbS/HbD/HbG migration region were interpreted in conjunction with

the sickling-solubility test and, when required, electrophoresis at acidic pH.

Cation-exchange high-performance liquid chromatography: CE-HPLC was performed using the Bio-Rad VARIANT II Haemoglobin Testing System with the Beta-Thalassaemia Short Program. Five microlitres of EDTA-anticoagulated whole blood were diluted in 1 mL of the manufacturer-provided haemolysis reagent. Haemoglobin fractions were separated on a weak cation-exchange cartridge using a programmed gradient of phosphate buffers with differing pH and ionic strength at a flow rate of approximately 2 mL/min. Eluted fractions were detected photometrically at 415 nm, with a secondary wavelength of 690 nm for background correction. Each analysis required approximately 6.5 minutes. The system generated a chromatogram showing retention times, peak areas and percentages of individual haemoglobin fractions. Prespecified retention-time windows included HbF at 0.98–1.20 minutes, HbA₀ at 1.90–3.10 minutes, HbA₂/E at 3.30–3.90 minutes, HbD at 3.90–4.30 minutes, HbS at 4.30–4.70 minutes and HbC at 4.90–5.30 minutes.

Diagnostic interpretation and outcome measures: A presumptive phenotypic diagnosis was established by integrating clinical history, transfusion status, erythrocyte indices, peripheral-smear findings, sickling-solubility results, electrophoretic migration and HPLC retention time and peak percentage. The principal outcomes were agreement between the two methods regarding haemoglobinopathy classification and differences in the measured percentages of HbA,

HbA₂, HbF and HbS. Discordant or indeterminate patterns were reviewed using supplementary laboratory findings, family studies and, in selected complex cases, molecular testing.

Statistical analysis: Analyses were performed using SPSS 20.0. Continuous variables were summarized as mean $\pm$ standard deviation, and categorical variables as frequencies and percentages. Because both techniques were applied to the same specimens, between-method differences in haemoglobin fractions were evaluated using the paired Student's t-test or Wilcoxon signed-rank test, as appropriate. Correlations were assessed using Pearson's or Spearman's coefficient. Categorical agreement was expressed as overall concordance and Cohen's kappa coefficient, with McNemar's test used to assess paired diagnostic disagreement. A two-sided p value <0.05 was considered statistically significant.

RESULTS

Of the 805 participants initially evaluated, seven (0.9%) were excluded because of a recent blood transfusion, leaving 798 participants for analysis. CE-HPLC identified an abnormal haemoglobin pattern in 635 participants (79.6%), while 163 (20.4%) had a normal adult haemoglobin pattern. The high proportion of abnormalities reflects the clinically selected, referral-based study population and does not represent community prevalence [Table 1].

Table 1: Participant recruitment, eligibility and final diagnostic classification.

Study stage	Number	%
Participants initially evaluated	805	100
Excluded because of blood transfusion during the preceding four months	7	0.9 ¹
Participants included in the comparative analysis	798	99.1 ¹
Haemoglobinopathy detected	635	79.6 ²
No abnormal haemoglobin detected	163	20.4 ²

¹Percentage calculated using 805 enrolled participants as the denominator. ²Percentage calculated using 798 analysed participants as the denominator. Both CE-HPLC and alkaline agarose-gel electrophoresis were performed in all 798 included participants.

Exact phenotype agreement between CE-HPLC and alkaline agarose-gel electrophoresis was observed in 790 of 798 cases (99.0%), corresponding to a multicategory Cohen's κ of 0.982 (p<0.001). Complete concordance was observed for normal patterns (163/163), HbAS (484/484), HbSS (101/101), β -thalassaemia major/intermedia (2/2),

HbS/ β -thalassaemia (4/4), HbAE (11/11), HbEE (2/2), HbSE (3/3), HbAJ Meerut (6/6) and HPFH (2/2). Among 16 β -thalassaemia-trait cases, 12 were correctly identified. The remaining disagreements involved four β -thalassaemia-trait, one HbAD, two HbDD and one Hb Lepore cases [Table 2].

Table 2: Phenotype-wise concordance between CE-HPLC and alkaline agarose-gel electrophoresis.

CE-HPLC phenotype	Number (%)	Concordant electrophoresis diagnosis	Discordant diagnosis	Exact concordance (%)
Normal haemoglobin pattern	163 (20.4)	163	0	100
Sickle cell trait (AS)	484 (60.7)	484	0	100
Sickle cell anaemia (SS)	101 (12.7)	101	0	100
Beta-thalassaemia trait (BTT)	16 (2.0)	12	4	75
Homozygous beta-thalassaemia ($\beta\beta$)	2 (0.3)	2	0	100
Sickle-beta-thalassaemia (S β)	4 (0.5)	4	0	100
Haemoglobin E trait (AE)	11 (1.4)	11	0	100
Homozygous haemoglobin E (EE)	2 (0.3)	2	0	100
Sickle-haemoglobin E disease (SE)	3 (0.4)	3	0	100

Haemoglobin J Meerut trait (AJ)	6 (0.8)	6	0	100
Haemoglobin D trait (AD)	1 (0.1)	0	1	0
Homozygous haemoglobin D (DD)	2 (0.3)	0	2	0
Haemoglobin Lepore	1 (0.1)	0	1	0
Hereditary persistence of fetal haemoglobin	2 (0.3)	2	0	100
Total	798 (100.0)	790	8	99

CE-HPLC, cation-exchange high-performance liquid chromatography; κ , Cohen's kappa coefficient. Percentages may not total exactly 100 because of rounding.

Among the 635 participants classified as having a haemoglobinopathy by CE-HPLC, electrophoresis identified 631 as abnormal and reported four as normal. All 163 CE-HPLC-normal cases were also classified as normal by electrophoresis, with no false abnormal classifications. Overall agreement was

794/798 (99.5%), and intermethod agreement was near-perfect ($\kappa=0.985$, $p<0.001$). Exact McNemar testing showed no statistically significant systematic difference in binary classification between the two methods ($p=0.125$) [Table 3].

Table 3: Agreement between CE-HPLC and alkaline agarose-gel electrophoresis for binary classification of abnormal and normal haemoglobin patterns.

CE-HPLC classification	Abnormal pattern on electrophoresis	Normal pattern on electrophoresis	Total
Abnormal haemoglobin pattern	631	4	635
Normal haemoglobin pattern	0	163	163
Total	631	167	798

Overall agreement: 794/798 (99.5%); Cohen's $\kappa=0.985$, $p<0.001$. Exact McNemar's test: $p=0.125$.

Eight cases showed phenotype-level discordance. Two HbDD cases were interpreted as HbSS and one HbAD case as HbAS by alkaline electrophoresis because HbD and HbS migrate within the same S/D/G zone at alkaline pH. One Hb Lepore case was classified as HbAE because of overlapping electrophoretic mobility. In addition, four β -

thalassaemia-trait cases were reported as normal by electrophoresis, most likely because mild HbA₂ elevation was insufficiently resolved or quantified by densitometry. CE-HPLC differentiated these cases using retention windows, peak morphology and quantitative haemoglobin fractions [Table 4].

Table 4: Nature and probable analytical basis of discordant haemoglobinopathy classifications.

CE-HPLC classification	Number	Electrophoretic interpretation	Principal analytical explanation
Homozygous haemoglobin D	2	Sickle cell anaemia	HbD and HbS co-migrated in the S/D/G region at alkaline pH
Haemoglobin D trait	1	Sickle cell trait	HbD and HbS were not reliably differentiated by alkaline electrophoretic mobility alone
Haemoglobin Lepore	1	Haemoglobin E trait	Overlapping or closely positioned electrophoretic fractions complicated identification
Beta-thalassaemia trait	4	Normal haemoglobin pattern	Mild elevation of HbA ₂ was not reliably detected or quantified by gel densitometry
Total	8		

CE-HPLC provided separate retention windows for HbD and HbS, whereas interpretation of electrophoretic bands required correlation with the sickling-solubility test and, where necessary, electrophoresis at acidic pH.

Among 163 participants with normal patterns, mean HbA was lower by CE-HPLC than by electrophoresis ($86.90\pm3.40\%$ versus $96.50\pm0.90\%$; mean difference -9.60 percentage points; $p<0.001$), with weak correlation between methods ($r=0.160$). Mean HbA₂ was $2.80\pm0.32\%$ by CE-HPLC and $3.20\pm0.37\%$ by

electrophoresis (difference -0.40 ; $r=0.870$; $p<0.001$). Mean HbF was also lower by CE-HPLC ($0.78\pm0.88\%$ versus $2.10\pm1.76\%$; difference -1.32 ; $r=0.968$; $p<0.001$). HbF demonstrated the greatest relative variability, with coefficients of variation of 112.7% and 82.6%, respectively [Table 5].

Table 5: Comparison of haemoglobin-fraction measurements by CE-HPLC and alkaline agarose-gel electrophoresis among participants with normal haemoglobin patterns.

Haemoglobin fraction	CE-HPLC mean $\pm$ SD (%)	Electrophoresis, mean $\pm$ SD (%)	Mean difference ¹	CV: CE-HPLC/ electrophoresis (%)	Correlation coefficient (r)	p value ²
HbA	86.90 ± 3.40	96.50 ± 0.90	-9.60	3.8/0.95	0.16	<0.001
HbA ₂	2.80 ± 0.32	3.20 ± 0.37	-0.40	11.1/11.4	0.87	<0.001
HbF	0.78 ± 0.88	2.10 ± 1.76	-1.32	112.7/82.6	0.968	<0.001

¹Mean difference calculated as CE-HPLC minus electrophoresis. ²Paired p values were calculated from the thesis-reported means, standard deviations and within-subject correlations; they should be confirmed against participant-

level data. CV, coefficient of variation; HbA, adult haemoglobin; HbA₂, minor adult haemoglobin; HbF, fetal haemoglobin; SD, standard deviation.

In participants with normal haemoglobin patterns, HbA₂ was significantly lower by CE-HPLC than by electrophoresis (2.80±0.32% versus 3.20±0.37%; $r=0.870$; $p<0.001$). A similar difference was observed in HbAS cases (3.60±0.37% versus 4.00±0.48%; $r=0.300$; $p<0.001$). Conversely, no significant difference was found in HbS/β-

thalassaemia (5.40±0.66% versus 5.30±0.33%; $r=-0.780$; $p=0.845$) or β-thalassaemia trait (4.96±0.58% versus 5.10±0.93%; $r=0.870$; $p=0.292$). In β-thalassaemia trait, CE-HPLC showed lower relative variability than electrophoresis (CV 11.8% versus 18.3%) [Table 6].

Table 6: Subgroup comparison of HbA₂ quantification by CE-HPLC and alkaline agarose-gel electrophoresis.

Diagnostic group	Number	CE-HPLC HbA ₂ , mean ± SD (%)	Electrophoresis HbA ₂ , mean ± SD (%)	Mean difference ¹	CV: CE-HPLC/electrophoresis (%)	r	Paired p value ²
Normal haemoglobin pattern	163	2.80 ± 0.32	3.20 ± 0.37	-0.40	11.1/11.4	0.87	<0.001
Sickle cell trait	484	3.60 ± 0.37	4.00 ± 0.48	-0.40	10.2/11.9	0.3	<0.001
Sickle-beta-thalassaemia	4	5.40 ± 0.66	5.30 ± 0.33	0.1	12.0/6.2	-0.780	0.845
Beta-thalassaemia trait	16	4.96 ± 0.58	5.10 ± 0.93	-0.14	11.8/18.3	0.87	0.292

¹Mean difference calculated as CE-HPLC minus electrophoresis. ²Paired p values were recalculated from rounded summary statistics and should therefore be regarded as approximate until verified using the original paired observations. The difference in HbA₂ was statistically significant in normal participants and those with sickle cell trait, but not in the small sickle-beta-thalassaemia or beta-thalassaemia-trait groups.

The distribution of major haemoglobin fractions was broadly comparable between the two methods. In HbAS, CE-HPLC and electrophoresis measured HbA at 57.0% and 61.0%, HbA₂ at 3.6% and 4.0%, HbF at 1.5% and 1.0%, and HbS at 32.1% and 33.0%, respectively. Corresponding values in HbSS were 7.8% and 7.0% for HbA, 3.5% and 3.9% for HbA₂, 19.0% by both methods for HbF, and 71.2% and 71.9% for HbS. In HbS/β-thalassaemia, HbS

predominated (66.0% versus 66.7%), with raised HbF (22.0% versus 19.0%) and HbA₂ (5.4% versus 5.3%). β-Thalassaemia-trait cases showed elevated HbA₂ (4.96% versus 5.10%), while β-thalassaemia major/intermedia showed markedly elevated HbF (34.3% versus 34.5%). Most differences were small, although the significant HbA₂ difference in HbAS was consistent with Table 6 (Table 7).

Table 7: Comparative quantification of major haemoglobin fractions within common haemoglobinopathy phenotypes.

Phenotype	Fraction	CE-HPLC mean (%)	Electrophoresis mean (%)
Sickle cell trait (n=484)	HbA	57	61
	HbA ₂	3.6	4
	HbF	1.5	1
	HbS	32.1	33
Sickle cell anaemia (n=101)	HbA	7.8	7
	HbA ₂	3.5	3.9
	HbF	19	19
	HbS	71.2	71.9
Sickle-beta-thalassaemia (n=4)	HbA	8.3	8.1
	HbA ₂	5.4	5.3
	HbF	22	19
	HbS	66	66.7
Beta-thalassaemia trait (n=16)	HbA	83.4	84.5
	HbA ₂	4.96	5.1
	HbF	0.8	1.8
	HbS	11.8	18.3
Homozygous beta-thalassaemia (n=2)	HbA	57	61
	HbA ₂	4.9	5.5
	HbF	34.3	34.5
	HbS	11.8	18.3

HbA, adult haemoglobin; HbA₂, minor adult haemoglobin; HbF, fetal haemoglobin; HbS, sickle haemoglobin.

CE-HPLC required approximately 5 µL of blood diluted in 1 mL of reagent and produced a chromatogram in approximately 6.5 minutes per sample. The Bio-Rad Variant II system permitted

automated loading of up to 100 samples, peak integration and separation of HbS and HbD into distinct retention windows. In contrast, alkaline electrophoresis required red-cell washing,

preparation of haemolysate, approximately 30 minutes of migration and subsequent fixation, staining, destaining, drying and densitometric interpretation, with approximately 10 samples processed per gel. Electrophoresis was less expensive

but more labour-intensive and could not reliably differentiate variants co-migrating within the S/D/G region. Both methods had limitations in differentiating HbE from HbA₂, necessitating complementary testing in uncertain cases [Table 8].

Table 8: Comparative analytical workflow and operational characteristics of CE-HPLC and alkaline agarose-gel electrophoresis.

Characteristic	CE-HPLC	Alkaline agarose-gel electrophoresis
Instrument	Bio-Rad VARIANT II	Maestro 101 Biotech-Fischer
Sample preparation	5 µL whole blood diluted in 1 mL haemolysis reagent	Separation and repeated saline washing of erythrocytes followed by haemolysate preparation
Analytical principle	Separation by cation exchange and retention time	Separation according to electrophoretic mobility at alkaline pH
Approximate analytical time	6.5 minutes per sample	30-minute electrophoretic migration, excluding fixation, staining, destaining and drying
Sample-loading capacity	Up to 100 samples per automated sequence	Approximately 10 samples per agarose gel
Quantification	Automated peak-area calculation	Densitometric measurement of stained bands
Differentiation of HbS and HbD	Separate S and D retention windows	Co-migration in the S/D/G region
HbA ₂ quantification	Automated and relatively precise	Greater operator and band-resolution dependence
HbE and HbA ₂	Co-elute in the HbA ₂ /E window	Co-migrate at alkaline pH
Detection of HbJ Meerut	Presumptive identification in the P3 window	Fast-moving band ahead of HbA
Cost and technical requirement	Higher reagent and equipment cost; requires trained chromatogram interpretation	More economical but labour-intensive
Overall role	Rapid screening and quantitative fraction analysis	Useful complementary method for confirming migration patterns

CE-HPLC, cation-exchange high-performance liquid chromatography; HbS, haemoglobin S; HbD, haemoglobin D; HbE, haemoglobin E; HbA₂, haemoglobin A₂; S/D/G region, alkaline electrophoretic zone shared by HbS, HbD and HbG.

DISCUSSION

The present study demonstrated excellent concordance between CE-HPLC and alkaline agarose-gel electrophoresis for haemoglobinopathy detection. Among 798 evaluable participants, 635 (79.6%) had an abnormal CE-HPLC pattern; this high proportion reflects referral enrichment and should not be interpreted as population prevalence. Binary classification agreed in 794/798 cases (99.5%; $\kappa=0.985$, $p<0.001$), and exact phenotype agreement was 790/798 (99.0%; $\kappa=0.982$, $p<0.001$). These results accord with contemporary Indian studies by Iyer et al., Mondal et al., and Warghade et al., in which CE-HPLC provided rapid recognition of common and uncommon phenotypes across cohorts ranging from 25,297 to 119,336 samples.^[11-13] Nadkarni et al., however, showed through combined HPLC, electrophoresis and molecular testing of 31,075 referred individuals that considerable genotypic diversity may underlie apparently similar phenotypes.^[14] Thus, the present κ values demonstrate near-perfect method agreement, not proof that either phenotypic method is invariably correct.

The four phenotype-specific discrepancies were clinically informative. Alkaline electrophoresis classified two HbDD cases as HbSS and one HbAD case as HbAS because HbD and HbS have similar mobility at alkaline pH. CE-HPLC separated them into characteristic retention windows, explaining its

superior discrimination in these cases. Recent diagnostic frameworks recommend by Lee et al., interpreting retention time together with peak area, red-cell indices and clinical or family information.^[15] In a 2021 comparison covering 102 variant types, Xu et al., detected 84 (82.4%) by Variant II CE-HPLC and 85 (83.3%) by capillary electrophoresis, confirming that these techniques are complementary rather than universally definitive.^[16] Contemporary southern-Indian data by Kuppusamy et al., likewise demonstrate that HPLC can recognize HbD, HbE, HbJ Meerut, Hb Lepore and compound heterozygous states, although uncommon patterns remain challenging.^[17]

Significant method-dependent differences occurred despite the high qualitative agreement. In normal samples, mean HbA was 86.90% by CE-HPLC versus 96.50% by electrophoresis, HbA₂ was 2.80% versus 3.20%, and HbF was 0.78% versus 2.10% (all $p<0.001$). HbA₂ and HbF were strongly correlated ($r=0.870$ and 0.968), but correlation measures parallel variation rather than numerical agreement. Lower HPLC HbA may arise because HbA₂, HbF, glycated fractions and P1/P2 or unidentified peaks are integrated separately, whereas densitometry may incorporate poorly resolved minor fractions into the HbA band. The very high HbF coefficients of variation are also expected when concentrations approach the analytical detection limit. In a 2024 analytical comparison, Laksap et al., reported an HbA₂ /E correlation of $r=0.997$ between premier-

resolution HPLC and capillary electrophoresis, alongside 100% agreement for thalassaemia interpretation, while still emphasizing method-specific quantitation.^[18]

For β -thalassaemia trait, HbA₂ values were similar by CE-HPLC and electrophoresis (4.96% versus 5.10%; $p=0.292$; $r=0.870$), but electrophoresis missed four of 16 cases and showed greater variability (CV 18.3% versus 11.8%). This supports automated peak integration when HbA₂ elevation is mild. Nevertheless, borderline HbA₂ cannot independently exclude carrier status: molecular testing in a recent series by Noor et al., identified β -thalassaemia mutations in 58/123 (47.1%) individuals with HbA₂ values of 3.0–3.9%.^[19] A 2025 Gujarat study by Anandani et al., similarly highlighted HbE/ β -thalassaemia, Hb Lepore, HbJ Meerut and raised HbF as recurring interpretative challenges on HPLC.^[20] Moreover, HbE may co-elute with HbA₂, while modified HbS fractions can interfere with HbA₂ estimation.

Recent molecular studies reinforce these cautions. Among 4,783 chromatograms, Tashfeen et al., employed molecular testing to confirm HbD-Punjab and identify rare variants that could not be assigned reliably by CE-HPLC alone.^[21] A 2026 cohort by Singha et al., found that variants associated with increased HbA₂ and HbF had substantially greater odds of β -thalassaemia misinterpretation (OR 18.2, 95% CI 7.1–48.9).^[22] An integrated approach combining capillary electrophoresis by Puthiyaveettil et al., HPLC and mass spectrometry has also resolved rare variants with atypical migration or retention patterns.^[23] while a 10-year newborn-screening study by Campos Pereira et al., demonstrated that HPLC could distinguish HbD-Punjab from Hb Korle-Bu when their electrophoretic patterns overlapped.^[24] Operationally, the 6.5-minute automated HPLC cycle and batch capacity substantially exceeded the labour-intensive electrophoretic workflow. Therefore, CE-HPLC is supported as the preferred first-line method, but discordant, borderline or rare results should undergo orthogonal electrophoretic and, where clinically consequential, molecular confirmation.

Limitations: This single-centre study included a clinically selected referral population, limiting generalisability and preventing estimation of community prevalence. CE-HPLC was used as the comparator without molecular confirmation of every phenotype; therefore, concordance cannot be equated with definitive diagnostic accuracy. Several rare phenotypes had very small subgroup sizes. Formal Bland–Altman analysis, repeatability testing and comprehensive cost-effectiveness assessment were not performed, restricting evaluation of quantitative agreement and laboratory implementation.

CONCLUSION

CE-HPLC and alkaline agarose-gel electrophoresis showed near-perfect agreement for

haemoglobinopathy detection; however, CE-HPLC provided faster processing, automated quantification and superior separation of variants co-migrating at alkaline pH. Its advantages were particularly evident for HbD/HbS differentiation and recognition of mildly elevated HbA₂ in β -thalassaemia trait. Nevertheless, significant method-dependent differences in HbA, HbA₂ and HbF measurements and potential chromatographic co-elution indicate that CE-HPLC should not be interpreted in isolation. CE-HPLC is therefore recommended as the preferred first-line technique, with electrophoresis, clinical and haematological correlation, family studies and molecular testing used for borderline, rare or discordant patterns, thereby improving diagnostic confidence and supporting appropriate patient counselling.

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