Original Research Article

Comparative study of solubility and hemoglobin electrophoresis with high performance liquid chromatography (HPLC) for screening of hemoglobinopathies and thalassemia: Study from central India

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Abstract

Introduction: High prevalence of hemoglobinopathies is seen in central India, screening and genetic counseling are essential for early detection and management.

Aim: The retrospective study was performed at Regional hemoglobinopathy detection and management centre (RHDMC) Nagpur, central India to find out relative frequencies of hemoglobinopathies and thalassemia present using solubility test, hemoglobin electrophoresis and high performance liquid chromatography (HPLC) as screening method and to compare results of HPLC with solubility and electrophoresis.

Materials and methods: A total of 105,211 cases were screened for sickle cell disease (SCD) and sickle cell trait (SCT) by solubility test during the period of January 2003 to January 2014. Of these 105,211 samples, 60,000 samples which were solubility positive, with doubtful solubility and solubility negative but suspicious for hemoglobinopathy and thalassemia also the cases of anemia were studied by hemoglobin electrophoresis at alkaline pH 8.6. Of which 5,111 cases were further

studied by HPLC and results of HPLC were compared with combined solubility and Hb electrophoresis.

Results: Of 105,211 cases screened for hemoglobinopathy by solubility and electrophoresis, 12,979 (12.33%) were having sickle cell trait (SCT) and 3,062 (2.91%) were of sickle cell disease (SCD). Of 5,111 (100%) HPLC study cases, total SCD and SCT were 3,132 (61.27%) followed by 315 (6.16%) of beta-thalassemia trait and 264 (5.16%) cases of compound heterozygous for HbS and beta-thalassemia. Hemoglobinopathies E and D alone and its combination with HbS or beta-thalassemia were also found. Rare cases of HbD Iran, HbJ variant and HbQ India, Hb Abruzzo and delta-beta thalassemia were detected. Combined solubility and hemoglobin electrophoresis was effective for diagnosis of SCD and SCT when compared to HPLC with good agreement between two test by kappa statistics, however for detection of beta-thalassemia trait and for compound heterozygous for HbS and beta-thalassemia false negatives cases were more, chi square test showed highly significant *P* value <0.01.

Conclusion: Combined solubility and electrophoresis are simple and cost effective alternative to HPLC for screening large population with high prevalence of SCD when resources are limited but for beta-thalassemia screening HPLC is mandatory.

Key words

Hemoglobinopathy, Thalassemia, HPLC, Solubility, Hemoglobin electrophoresis.

Introduction

The hemoglobinopathies are autosomal recessive disorders, heterozygous, homozygous or genetic compound states resulting in clinically significant phenotypes of variable severity (thalassemia major, thalassemia intermedia, sickle cell syndromes, HbE syndromes). Heterozygous are symptom-free but presents with characteristic hematological features, often useful for their identification [1].

300,000 and 400,000 babies are born with a serious hemoglobin disorder each year and that up to 90% of these births occur in low- or middle-income countries [2]. Carriers of hemoglobinopathies are partially protected against morbidity and mortality of falciparum malaria and this has resulted in their higher prevalence in tropical countries. In India, they are responsible for the largest number of genetic disorders and hence are of great public health importance [3].

Of the several abnormal hemoglobin molecules, three which are widely prevalent in India include: HbS, HbE and HbD. The cumulative gene frequencies of these hemoglobins have been found to be 5.35% in India [4]. Carrier state for beta-thalassaemia in India varies from 1-17% with an average of 3.2% [2]. Vidarbha region of central India is having high prevalence of hemoglobinopathies specially sickle cell disease [2]. Heterozygote screening and genetic counseling are essential for early detection and control of severe hemoglobinopathies and thalassemia [5].

The laboratory diagnosis of thalassemias and other hemoglobinopathies can be satisfactorily done by combined approach including detailed clinical history, hematological profile including complete blood counts (CBC), solubility and sickling test, Hb-electrophoresis, high performance liquid chromatography (HPLC), and DNA analysis. Family studies also play a crucial role in diagnosis in problematic cases [6].

Due to poor infrastructure of medical laboratories in developing countries including India also the cost involved interfere with diagnosis of hemoglobinopathies and thalassemia. Due to lack of knowledge regarding their prevalence, poor facilities for their

diagnosis and due to presence of only few centers for prenatal diagnosis have resulted in failure of community control of birth of these totally preventable dreadful genetic disorders [3].

The previous studies from central India have been published focusing on sickle cell disease. This is the first study from this region consist of HPLC data of hemoglobinopathies as well as thalassemia.

The aim of the present study was to find out the relative frequencies of hemoglobinopathies present in region using solubility, hemoglobin (Hb) electrophoresis and high performance liquid chromatography (HPLC) as a screening tool and to compare diagnostic utility of basic tests (solubility and Hb electrophoresis) over HPLC.

Materials and methods

This was a retrospective study, carried out at Regional hemoglobinopathy detection and management centre (RHDMC) of Vidarbha, central India during the period of January 2003 to January 2014.

A total of 105,211 cases were screened for sickle cell disease (SCD) and sickle cell trait (SCT) by solubility test during the period of January 2003 to January 2014. Informed consent from the cases was taken. Of these 105,211 samples, 60,000 samples which were solubility positive, with doubtful solubility and solubility negative but suspicious for hemoglonopathy and thalassemia also the cases of anemia were studied by hemoglobin electrophoresis at alkaline pH 8.6. Of which 5,111 cases were further studied by HPLC and results of HPLC were compared with combined solubility and Hb electrophoresis.

HPLC was done in solubility negative “AS” pattern cases, cases for prenatal diagnosis of hemoglobinopathies, cases suspected for beta-thalassemia and other hemoglobinopathies, family members of homozygous cases to rule out compound heterozygous state with beta-thalassemia or with other hemoglobinopathy. Majority of SCD cases were studied by HPLC for quantification of Hb fractions, to rule out compound heterozygous state with beta-thalassemia and also for initiation of therapy [7]. CBC was done in all HPLC studied cases and peripheral smears were done whenever necessary. DNA analysis was done in some cases and it was advised for problematic cases.

This study included referred to a clinical diagnostic laboratory for confirmation of clinically suspected cases of hemoglobinopathy or beta thalassemia. Family screening of index case, screening of all pregnant women and couples seeking advice for prenatal diagnosis of hemoglobinopathies and screening camps for hemoglobinopathies were done in urban as well as tribal area. Neonates and infants up to 1 year were not included in study. In patients requiring frequent blood transfusions, sampling was deferred for 3 months or done before next transfusion.

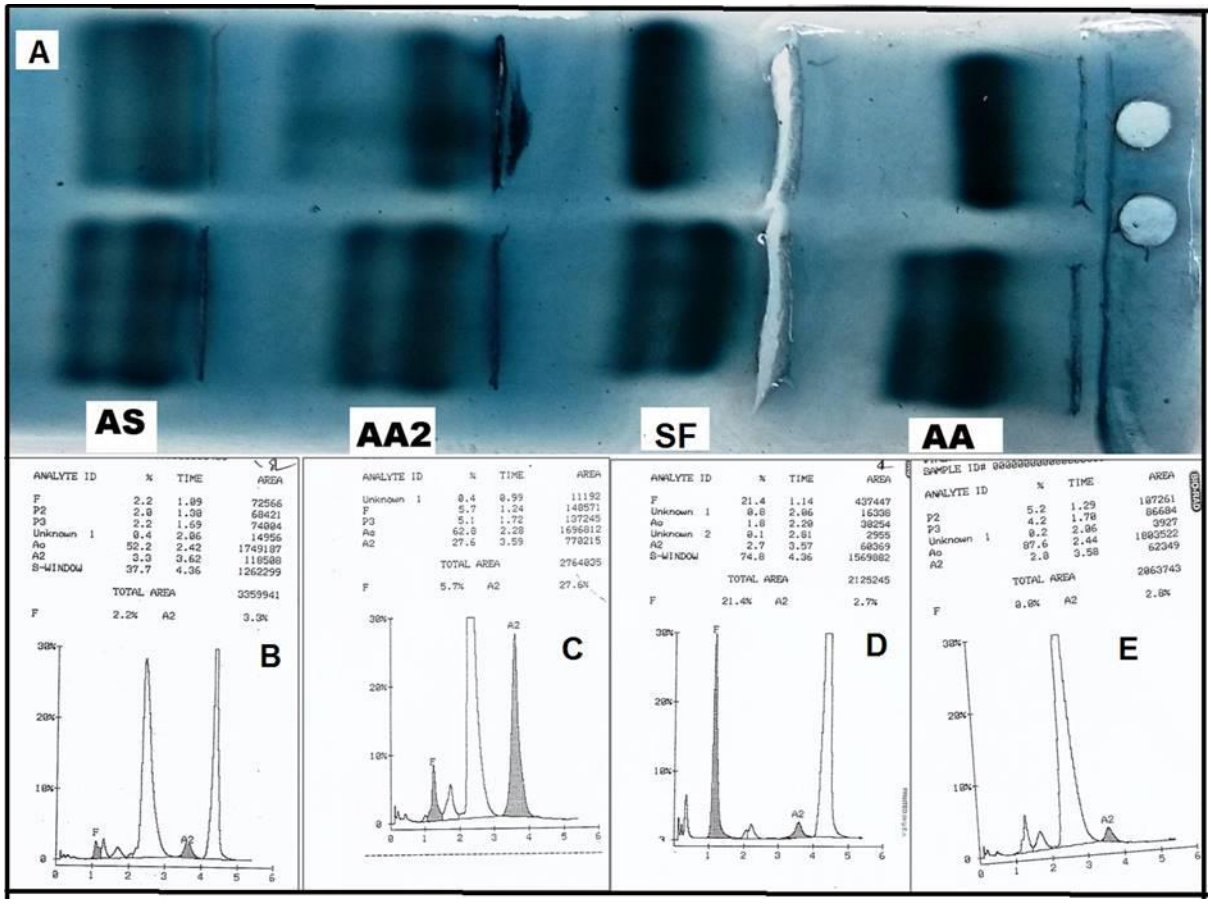
Solubility testing was done using high molar phosphate buffer and sodium diathionate as reducing agent [8]. Samples showing positive and doubtful solubility result were considered as positive for statistical analysis. Hemoglobin electrophoresis was done manually using alkaline Tris EDTA borate buffer at alkaline pH 8.6 on agarose gel slides and stained with amido black stain. Hb electrophoresis at alkaline pH could detect A, A2, F, S and D band [9]. As S and D band appears at the same position on haemoglobin electrophoresis at alkaline pH, positive solubility confirmed sickling status while negative solubility test is characteristic of HbD [10]. As densitometer was not available quantification of Hb fraction of bands separated by electrophoresis was not done (**Figure – 1A**).

HPLC was performed on BIO RAD variant [I] machine using beta thalassemia short program (Bio-Rad laboratories, Hercules, CA, USA). The different Hb variants were identified by using retention time windows which are specified for

these variants [11]. HPLC graphs of various hemoglobinopathies and thalassemias detected are shown in (**Figure 1 to 4**). CBC and RBC parameters were measured using cell analyzer (Sysmex XX-21 Tokyo, Japan).

Results of combined solubility and electrophoresis were compared with HPLC. Sensitivity, Specificity, positive predictive value, Negative predictive value was calculated. The statistical analysis was done by using Mac Nemar chi square test to compare the results of the two tests. *P* value was calculated, *P* value <0.01 suggested the two test give different results while *P* value >0.05 suggested two tests give same results. Also agreement between two tests was calculated by using kappa statistics. Chi square test was used to find the proportion of false negative. The analysis was done by using statistical software STATA 13 and Microsoft Excel.

Figure - 1: A] Manual electrophoretic separation of hemoglobin bands at the PH 8.6 on agarose gel also stained with amido black stain shows AS, AA2, SF (faint F band), AA bands.
Corresponding HPLC graphs run on Bio-Rad variant I machine using beta-thal short program shows A] Sickle cell trait (AS), B] HbE trait (AE), C] Sickle cell disease (SS), D] Normal case (AA).



All procedures performed in studies involving human participants were in accordance with the ethical standards of the institution.

Results

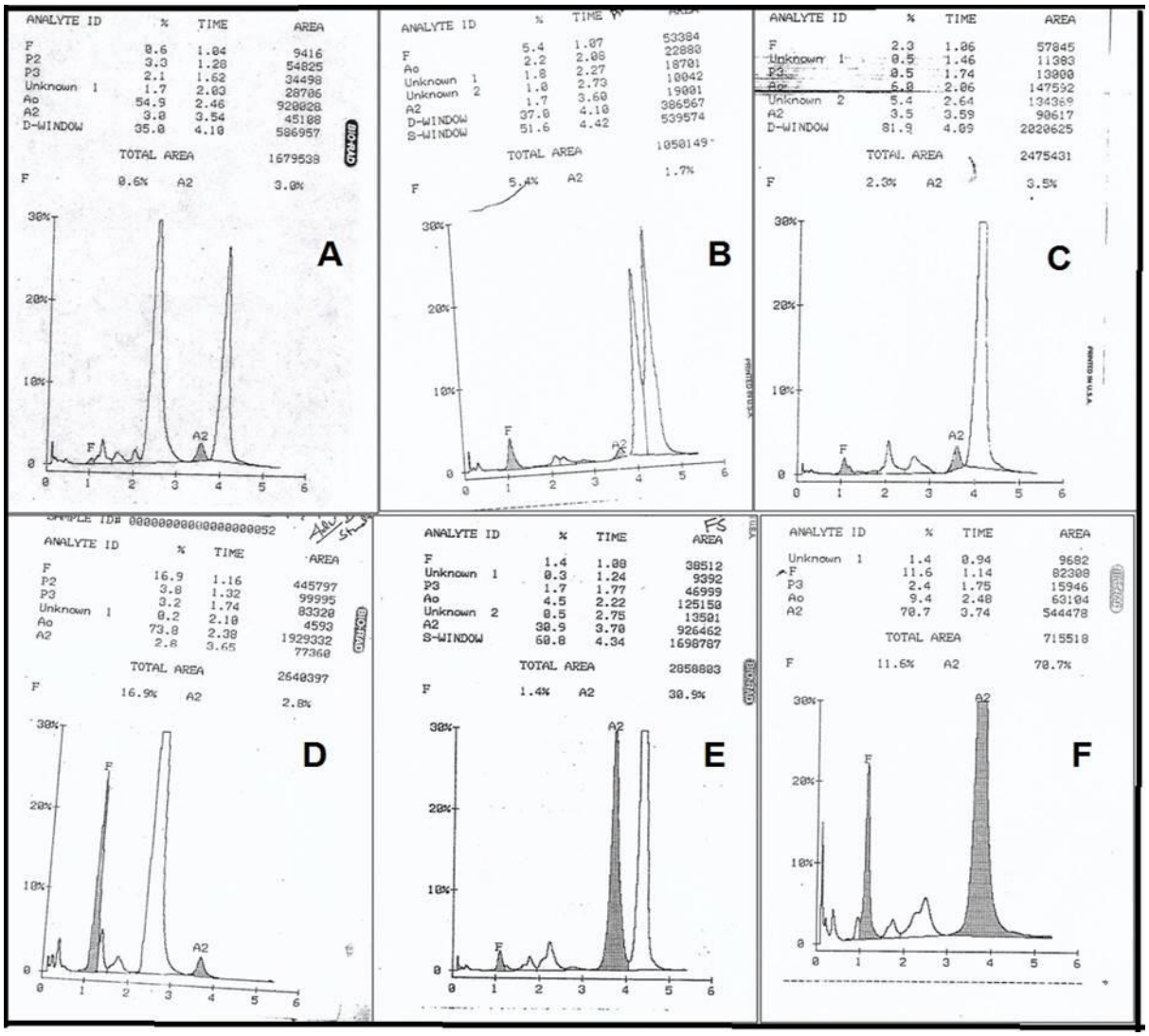
A total of 105,211(100%) cases were screened for sickle cell hemoglobinopathy by solubility and Hb electrophoresis, of which 12,979 (12.33%) were carriers (SCT) and 3,062 (2.91%) were having sickle cell disease (SCD).

Of 5,111(100%) HPLC studied cases which were actually included in the study, 1,236 (24.18%) were males and 3,875 (75.81%) were females

with age ranging from 1 year to 90 years with mean age of 21 years. The preponderance of young female cases was due to antenatal hemoglobinopathy screening.

Table - 1 shows distribution of the cases according to the type of hemoglobinopathies detected by HPLC with number of cases, Hb fractions in percentage with retention time (RT) of hemoglobin, Hb electrophoresis and solubility results.

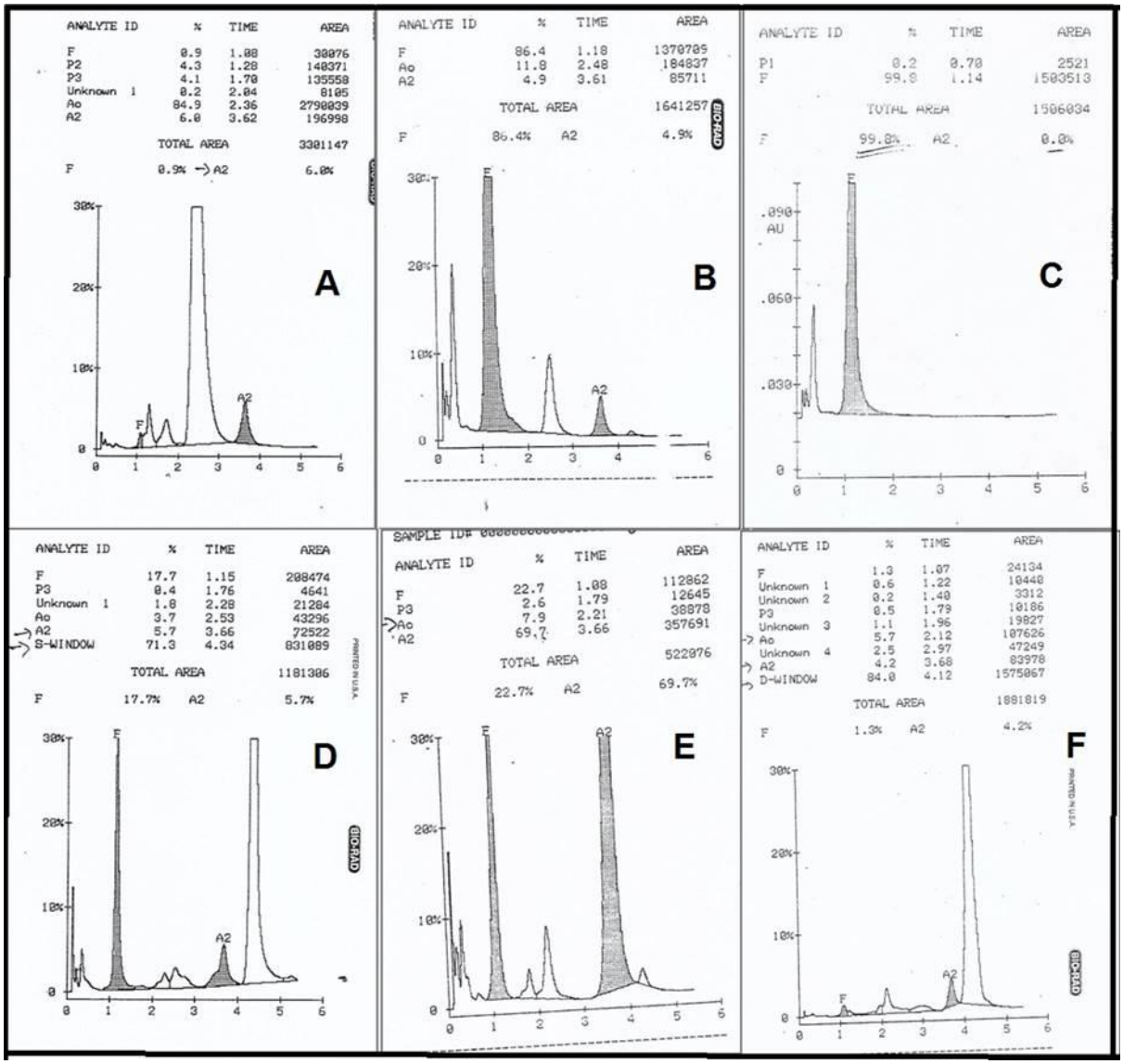
Figure - 2: HPLC graphs run on Bio-Rad variant I machine using beta-thal short program shows A] HbD trait (AD), B] Compound heterozygous for HbS and HbD (SD,) C] Homozygous D (DD), D] HPFH trait, E] Compound heterozygous for HbS and HbE, F] Homozygous E (EE).



Of 5,111 (100%) cases 1,163 (22.75%) cases showed normal AA pattern, while 3,948 (77.25%) cases showed abnormal pattern.

Sickle cell hemoglobinopathy (HbS) was present in high frequency with 1,710 (33.45%) of SCD and 1,422(27.82%) cases of SCT. Of 1,422 cases of SCT, associated alpha-thalassemia was suspected in 580 cases. Compound heterozygous cases of HbS with beta-thalassemia (Sβ) found in good frequency with 264 (5.16%) cases. Compound heterozygous cases of HbS with hemoglobinopathy HbE and HbD also found in the region with number of cases mentioned in table (**Table - 1**).

Figure - 3: HPLC graphs run on Bio-Rad variant I machine using beta-thal short program shows A] Beta-thalassemia trait, B] Homozygous for Beta-thalassemia (beta-thalassemia major), C] Homozygous for HPFH, D] Compound heterozygous for HbS and beta-thalassemia (Sβ0), E] Compound heterozygous for HbE and beta-thalassemia (Eβ thalassemia), F] Compound heterozygous for HbD and beta-thalassemia (Dβ thalassemia).



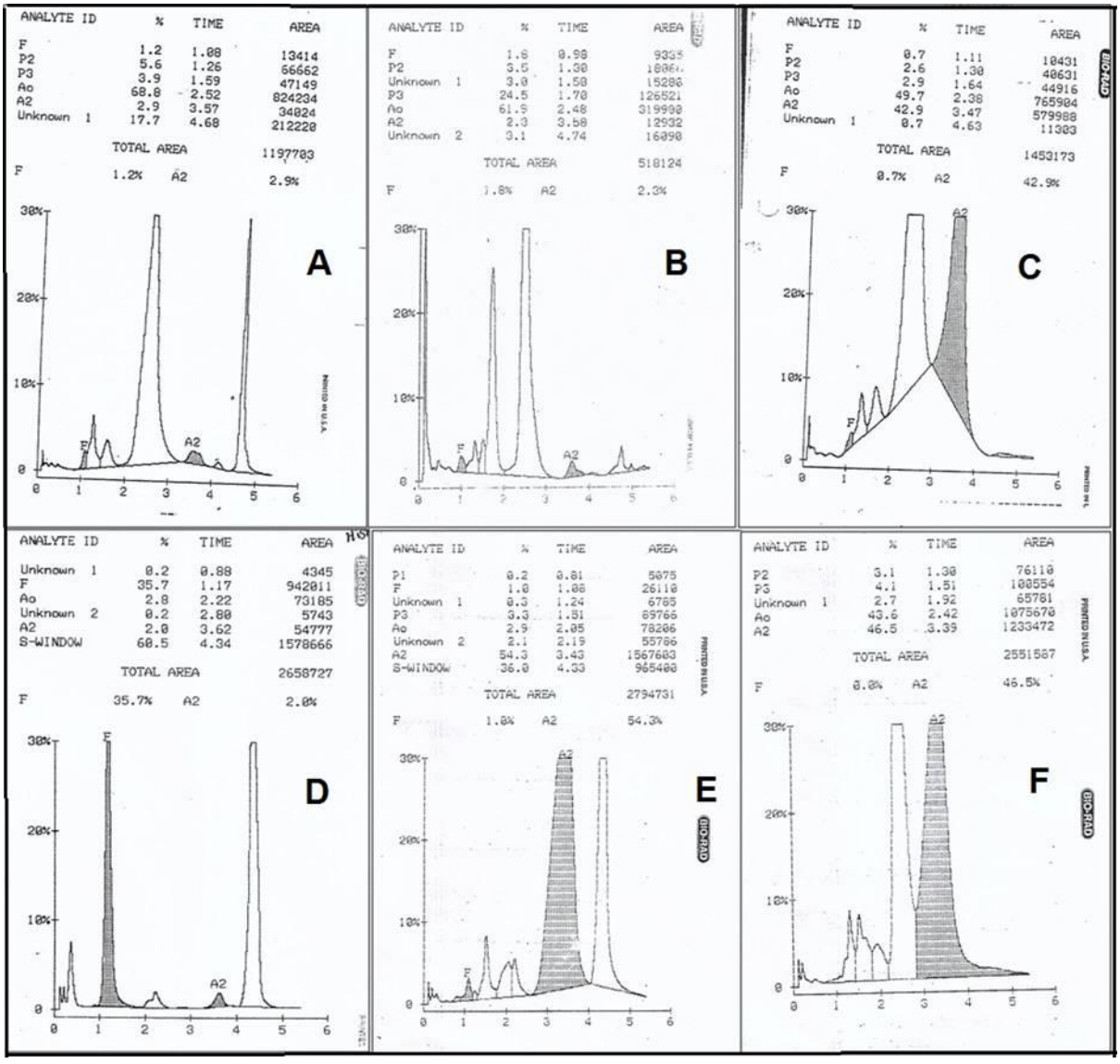
315(6.16%) cases of beta-thalassemia trait were diagnosed with 63 (1.23%) cases of homozygous for beta-thalassemia, cases of compound heterozygous for beta-thalassemia with HbE and HbD were also found. 63(1.23%) cases of HbE trait and 46 (0.90%) cases of HbD trait were diagnosed, cases homozygous for HbE and HbD were also present.

10 cases individually of heterozygous for hereditary persistence of fetal hemoglobin (HPFH) and compound heterozygous for HbS and HPFH (S\HPFH), along with 1 case of homozygous HPFH were found. Rare cases of delta beta thalassemia, HbD Iran trait, HbJ variant and HbQ India trait, Hb Abruzzo trait and compound heterozygous for HbS and Hb Abruzzo were also detected.

Mean hemoglobin level of SCD cases was 7.21±2.37 gm/dl. SCD cases showed variable percentage of HbF ranging from less than 25% upto 35%. Of 1,710 SCD cases, 1,395 cases showed HbF<25%, HbF values ranging 25-30%

was found in 185 cases and 130 cases showed HbF values between 30-35%. Some of the cases gave history of hydroxyurea therapy while some were not able to mention about it, however their parents were HbS trait, therefore they were included in SS category [7]. 10 cases of S/HPFH showed HbF percentage ranging between 25-37% with parents showing heterozygosity for HPFH.

Figure - 4: HPLC graphs run on Bio-Rad variant I machine using beta-thal short program shows A] HbQ India trait, B] HbJ variant trait, C] HbD Iran trait, D] Compound heterozygous for HbS and HPFH (HbS/HPFH), E] Compound heterozygous for HbS and Hb Abruzzo F] Hb Abruzzo trait.



SCT cases were mostly asymptomatic and were diagnosed during screening. SCT with associated alpha-thalassemia was suspected when HbS was <34% [12]. 580 cases were labeled as SCT with alpha-thalassemia with HbS ranging from 10%-34% with mean HbS of 29.74% based on trimodal distribution of HbS in SCT [12]. DNA studies were advised for confirmation.

Compound heterozygous for HbS and beta-thalassemia was diagnosed when along with HbS, the HbA2 percentage was more than 3.9%. $S\beta_+$ cases had HbA>HbA2 and $S\beta_0$ patients had Hb A2>HbA [6, 13]. Clinically $S\beta_0$ patients showed persistent splenomegaly at adulthood and frequent transfusion dependence as compared to SCD. In $S\beta_+$ patients symptoms were milder as

compared to SCD and $S\beta_0$ patients. Cases of compound heterozygous for HbS with HbE or HbD disease were presented with mild hemolytic anemia.

Cases with decreased Hb, decreased MCV and MCH, increased RBC count, normal RDW and microcytosis on peripheral blood, were screened for beta-thalassemia trait. Cut off for HbA2 was >3.9% [6, 14]. 315 patients were diagnosed as beta-thalassemia trait and their mean Hb was 10.5+/-5.43gm/dl, PCV 34.94+/-18.81%, MCV 68.19+/-9.26 fl, MCH 19.86+/3.53Pg, RDW 14.9+/-3.08, RBC count 4.99+/-1.09 $x10^6$/ ul and mean HbA2 of 5.28%, ranging from 3.9-9.9%

Table - 1: showing Hemoglobinopathies detected by HPLC with number of respective cases, Hb fractions in percentage with retention time (RT) of hemoglobin variant, Hb electrophoresis and solubility results.

Type of HB pathy detectedby HPLC	No. Of pts n=5,111 (100%)	Hemoglobin fraction percentage on HPLC						Pattern on Hb electro-phoresis and number of patients	Solubility results
		HbA% RT-2.04-2.84min	Hb F% RT -0.93-1.22min	HbA2% RT-3.52-3.68min	HbS% RT-4.27-4.62min	HbD% RT-3.92-4.30min	Other Hb% RT		
Normal	1,163 (22.75)	85.22± 7.78	0.98 ±1.20	2.74 ± 0.68	-	-	-	AA-1,161 AF-2	N-1,142 D-15 P-6
SCD	1,710 (33.45)	5.03± 9.3	20.13 ±7.72	3.10 ±1.17	74.10 ±11.83	-	-	SS-1,680 SF-25 F-2, AS-3	P -1,350 D-300 N - 60
SCT	1,422 (27.82)	53.76 ±5.9	1.41 ±1.3	3.19 ±0.87	35 ± 5.7	-	-	AS-1,419 AF-3	P -1,258 D-150 N -14
SCT with α-thal	580/1,422 Of SCT	56.79± 6.02	1.95± 1.4	3.28± 1.09	29.74± 4.06	-	-	AS-577 AF-3	P -500 D-72 N -8
S/HPFH	10(0.19)	4.13± 7.01	31.85 ± 5.15	2.9 ±1.05	62.73± 8.40	-	-	SF-8 SS-2	P-7 D-1 N-2
Sβ0	206(4.03)	3.49± 1.63	17.91 ± 7.19	6.99± 2.28	69.99± 9.82	-	-	SFA2-30 SF-49 SA2-2 SS-125	P-190 D-10 N-6
Sβ+	58(1.13)	11.45± 8.76	18.46 ± 7.60	5.57± 2.06	62.78± 10.07	-	-	SS-49 SF-3 ASA2-5 SFA2-1	P-51 D-6 N-1
β-Thal trait	315(6.16)	81.81± 7.06	2.39± 4.07	5.28± 1.27	-	-	-	AA2-50 AA-265	P-2 D-3 N-310

Type of HB pathy detectedby HPLC	No. Of pts n=5,111 (100%)	Hemoglobin fraction percentage on HPLC						Pattern on Hb electro-phoresis and number of patients	Solubility results
		HbA% RT-2.04-2.84min	Hb F% RT - 0.93-1.22min	HbA2% RT-3.52-3.68min	HbS% RT-4.27-4.62min	HbD% RT-3.92-4.30min	Other Hb% RT		
β-Thal Major (clinically)	42(0.82)	23.46± 13.43	67.74 ±34.30	3.85 ±1.37	-	-	-	AF-19 F-20 AFA2-3	N-41 D-1
β-Thal Intermedia (clinically)	21(0.41)	30.46± 25.11	64.11 ±27.20	4.4±2.49	-	-	-	AF-13 F-6 AFA2-2	N-21
HbD trait	46(0.90)	51.65± 9.02	1.47± 1.11	2.68 ± 0.65	-	35.32±6.68	-	AS-46	N-45, P-1
HbD Iran	1(0.01)	49.7	0.7	-	-	-	42.9 RT-3.47	AS-1	N-1
Homozygous D	4(0.09)	4.9± 1.13	4.9±6.29	2.4± 0.63	-	82.5 ±8.13	-	SS-5	N-5
Dβ Thal	1(0.01)	5.7	1.3	4.2	-	84	-	SS-1	N-1
SD	10(0.19)	2.44± 2.07	13.6 ±9.66	2.72± 0.79	39.9 ±10.16	44.6 ± 2.67	-	SS-10	P-10
HbE trait	63(1.23)	57.84± 12.84	2.59± 5.13	29.06 ±5.16	-	-	-	AA2-62 AA-1	N-62 P-1
Eβ Thal	8(0.15)	6.67± 3.50	20.97 ±11.94	70.31 ±9.56	-	-	-	FA2-7 A2-1	N-8
Homozygous E	1(0.01)	9.4	11.6	70.7	-	-	-	A2 thick band	N-1
SE	8(0.15)	4.2± 1.79	9.33 ±13.99	34.75 ±9.78	54.53 ±8.16	-	-	SA2-8	P-8
Heterozygous for δβ-Thal	1(0.01)	-	96	-	-	-	-	F-1	N-1
δβ Thal trait	4(0.07)	68± 2.05	20± 1.63	2.27 ± 0.12	-	-	-	AF-4	N-4

Type of HB pathy detected by HPLC	No. Of pts n=5,111 (100%)	Haemoglobin fraction percentage on HPLC					Other Hb% RT	Pattern on Hb electro-phoresis and No. of pts.	Solubility results
		HbA% RT-2.04-2.84min	Hb F% RT -0.93-1.22min	HbA2% RT-3.52-3.70min	HbS% RT-4.27-4.62min	HbD% RT-3.92-4.30min			
Hb J Trait	3(0.05)	67.05 ±7.70	2.6± 0.5	2.75 ±1.34	-	-	P3-22.75± 6.85 RT1.66-1.8	AA-3	N-2 D-1
Homozygous HPFH	1(0.01)	-	99.8	-	-	-	-	F-1	N-1
HPFH trait	10(0.1)	70.2± 1.12	20.4± 1.71	2.1	-	-	-	AF-8 AA-2	N-10
HbQ India trait	1(0.01)	70.3	-	3	-	-	19 RT-4.70	AD-1	N-1
Hb Abruzzo trait	1 (0.01)	43.6	-	-	-	-	46.5 RT-3.39	AF-1	N-1
HbS and Hb Abruzzo	1(0.01)	2.9	-	-	36	-	54.3 RT-3.43	SF-1	P-1

Footnote: HPLC- high performance liquid chromatography, SCD- sickle cell disease, SCT- sickle cell trait, Thal- Thalassemia, HPFH- heriditory persistence of fetal hemoglobin, N- negative, P- positive, D- doubtful, RT- Retension Time, Min- Minutes.

There were 63 (1.23%) cases of homozygous for beta-thalassemia from which 42(0.82%) cases behaved phenotypically as thalassemia major. Patients presented with failure to thrive and transfusion dependent anemia, thalassemic facies and marked splenomegaly. 21(0.41%) cases behaved phenotypically as thalassemia intermedia presented with anemia requiring no or occasional transfusion.

One case of homozygous for delta-beta thalassemia was detected having HbF 96% with absence of A and A2. Patient was having mild hemolytic anemia requiring occasional transfusion. Family screening revealed 4 cases of delta-beta thalassemia trait having increased HbF and normal to decreased HbA2 [15]. DNA analysis of suspected case of homozygous for delta-beta thalassemia was done; using a gap PCR based strategy for the 3 known HbF determinants in Indian population (HPFH-3, Indian GyAy ($\delta\beta^0$) thalassemia, Asian- Indian Inversion) and It was found that the case was homozygous for Indian GyAy ($\delta\beta^0$) thalassemia inversion. This mutation is caused by a major rearrangement including an inversion of sequence between the 3' end of the Ay gene and the IVS II region of the β globin gene followed

by a deletion (total 8.5 kb) of the flanking DNA sequence. Family studies of this case showed that his parents and siblings were heterozygous for this mutation.

Table - 2: Showing comparison of combined solubility and electrophoresis with HPLC results statistical analysis.

Statastical Results	HbEp and solubility in SCD %	HbEp and solubility in SCT %	solubility for screening SCD and SCT %	HbEp for detection of Thal Trait %	HbEp for detection of HbS with βThal %
Sesitivity	99.70	99.78	97.59	15.87	14.39
Specificity	92.76	99.89	98.20	99.87	99.85
Positive Predictive Value	87.39	99.71	99.11	89.28	84.44
Negative Predictive Value	99.84	99.91	95.22	94.75	95.53
Agreement between two tests by Kappa statastics	99.40	99.86	97.78	__	__
Mac nemar's Chi square test for comparison of two tests with p value	<0.01	>0.05	<0.01	__	__
Chi square test for comparison of false negative cases in two tests	__	__	__	<0.01	<0.01

Footnote: HbEp- Hemoglobin electrophoresis at alkaline pH 8.6., SCD-sickle cell disease, SCT-sickle cell trait, HBS with βThal- compound heterozygous cases of HbS and beta-thalassemia.

Cases of HbE and HbD trait cases were mostly asymptomatic or having mild anemia. HbE cases showed HbE% ranging from 16.5- 36% with mean HbE 29.06% while HbD trait cases showed HbD% ranging between 22-46% with mean HbD 35.33% [16, 17].

1 case of HbD Iran trait was detected having HbA2 42.9% with RT of 3.47. Solubility test was negative and Hb electrophoresis showed band in S/D/G position [18]. 3 cases of HbJ variant were detected. Variant hemoglobin eluted in P3 window with percentage varying from 17-27% [19].

1 case of homozygous HPFH was suspected, patient was asymptomatic with Hb of 10g/dl and HbF 99.8%. 10 cases of HPFH trait were suspected, HbF ranged from 16-27%.

One case of HbQ India was observed. Hemoglobin separated with retention time of

4.69 min which matched with retention time of HbQ India and variant hemoglobin was 19%. On electrophoresis the band separated in S position and solubility test was negative [18].

One asymptomatic case of compound heterozygous for HbS and Hb Abruzzo was detected, where father was having Hb Abruzzo trait and mother was having SCT. Hb Abruzzo is a high oxygen affinity variant formed by substitution of arginine for histidine at the Cd143, that is, CAC->CGC. Hb Abruzzo eluted in A2 window on HPLC with RT of 3.39 min, appeared in the position of F band on Hb electrophoresis and clinically presented with relative polycythemia and erythrocytosis in heterozygous. DNA sequencing of the father and child confirmed the diagnosis [20].

Statistical analysis of comparison of results of combined solubility and electrophoresis with HPLC is given in **Table - 2**.

When solubility test was done along with electrophoresis in detection of SCD and results were compared to HPLC, agreement between two tests by Kappa analysis was-99.40%. Mac Nemar chi square test was used for statistical analysis, *P* value obtained was<0.01 which was significant, suggested that both of the tests do not give same results for diagnosis of SCD however as the agreement between two test was good, suggesting combined solubility and Hb electrophoresis at alkaline pH was effective for diagnosis of SCD compared to HPLC.

When electrophoresis was done along with solubility testing for detection of SCT cases with comparing its results HPLC, agreement between two tests by kappa analysis was 99.86%. Mac Nemar chi square test was used for statistical analysis, *P* value obtained was>0.05 which was not significant, suggesting that electrophoresis in combination with solubility gives same result to diagnose SCT cases when compared with HPLC.

To find Utility of solubility in detecting of burden of SCD and carriers in the society as compared to HPLC, agreement by Kappa statistics was 97.78%. Mac nemar chi square test used for statistical analysis, *P* value was significant <0.01 suggested that solubility test was not as effective as HPLC but as the agreement between two test was good, solubility test was easy and cost effective for screening large population, though it cannot differentiate between disease and carriers it is useful in mass screening so that further electrophoresis was carried out to confirm the disease or the carriers.

While comparing utility of electrophoresis in detection of beta-thalassemia trait and compound heterozygous cases of HbS and beta thalassemia, it was found that electrophoresis gave more false negative cases compared to HPLC. Chi square test was used for statistical analysis with *P* value <0.01 suggested that electrophoresis gives more false negative cases as compared HPLC.

Discussion

Sickle cell hemoglobinopathy and beta-thalassemia trait were common hemoglobinopathy present in the study which was carried out in Vidarbha, central India. HbE and HbD hemoglobinopathies also present in region, with rare variants as delta-beta thalassemia, HbJ variant, HbQ India, Hb Abruzzo were also detected. However distribution of hemoglobinopathies is not uniform in all regions of India. Beta-thalassemia are common in North India [18], in Assam HbE gene is prevalent [21]. In Orissa and South Gujarat beta-thalassemia trait and sickle cell traits are common [22, 23], while in West Bengal and South India beta-thalassemia traits and HbE traits are found [24, 25].

In SCD cases mean HbF was 20.3% which was ranging from less than 25% up to 35% with mean Hb-7.21±2.37gm/dl. These findings suggest that patients were having moderate anemia with high HbF as it is found in Arab-Indian haplotype [26]. Rahimi, et al. studied different sickle haplotypes

in Iran patients [26]. Arab-Indian was the most prevalent haplotype in their study population, accounting for 51.1%. In the homozygous SS Arab-Indian haplotype mean HbF was 30.40%, while in AS cases the HbF% was only 1.20 matching with our AS cases with mean HbF of 1.41 [26].

580 of 1,422 SCT cases were labeled as SCT associated with alpha-thalassemia with HBS-29.74±4.06% and HBA-56.79±6.02%. Phenotypic expression of sickle-cell anemia, linked to the Arab-India haplotype and expressing similar levels of HbF is not uniformly mild in India and coinherited alpha-thalassemia is a powerful and additional factor in the Indian subcontinent that play a synergistic role in ameliorating the severity of the sickle cell disease. Therefore it is necessary to document alpha thalassemia based on HPLC findings of trimodal distribution of HBS [12, 27].

Hb variant such as HbD Iran, Hb Abruzzo were identified based on their unique HPLC, Hb electrophoresis and CBC findings. HbD Iran, Hb Abruzzo along with common variant HbE and HbA2 elute in A2 window on HPLC. HbA2 percentage in HbE trait and beta-thalassemia trait is <40% and it appears at A2 band position on Hb electrophoresis at alkaline pH. In HbD Iran and Hb Abruzzo percentage of hemoglobin fraction in heterozygous is above >40%, HbD Iran gives band in SDG region and Hb Abruzzo gives band in F region on Hb electrophoresis at alkaline pH. As Hb Abruzzo is high oxygen affinity Hb variant, cases shows relative polycythemia and erythrocytosis on CBC, while it is not observed in HbD cases. However, DNA analysis is required to confirm such cases [6, 11, 20].

While comparing utility of combined solubility and Hb electrophoresis at alkaline pH for detection of SCD and SCT cases, combined solubility and electrophoresis was having high sensitivity and specificity for diagnosis of SCT and SCT when compared to HPLC with agreement between two test by kappa statistics 99.86% and 99.40% respectively. Hb electrophoresis at alkaline pH shows S, D, G bands in the same region this problem was solved, when solubility was used along with electrophoresis (**Table - 2**).

When Solubility test was used to screen for HbS and results were compared with HPLC, agreement between two test by Kappa statistics was 97.78% suggesting solubility test was effective for screening (**Table - 2**). Though it cannot differentiate between disease and carrier state, it is useful in mass screening to detect HbS so that further Hb electrophoresis and HPLC can be carried out to confirm the cases particularly in remote areas where other facilities are not available [8].

Beta-thalassemia cases alone and associated with other hemoglobinopathies were found with good frequency in our study with 315(6.16%) cases of beta-thalassemia trait and 264(5.16%) cases of HbS with beta thalassemia (Sβ). Hb electrophoresis was unable to detect small percentage of A2 <10% while HPLC could detect. HPLC usually provides accurate quantification of HbA2 and is therefore suitable for the diagnosis of beta-thalassemia trait [10, 28].

As far as HbD and HbE were concerned combined solubility and electrophoresis as well as HPLC could give satisfactory diagnosis. As there was small sample size of HbE and HbD statistical analysis was not done in these cases.

Though our regional centre for hemoglobinopathy detection sometimes due to limited availability of HPLC reagents and also due to its higher cost we could not do HPLC of all cases and then we had to depend on manual electrophoresis. In this study we have run Hb electrophoresis on manually prepare agarose gel slides on which different Hb bands get separate but for getting sharp bands we can use commercially available better quality gel strips

however due to it cost of test would increase. In our study we have not done iron profile of suspected cases for beta-thalassemia trait with HbA2 between 3.5%-3.9%, however for such cases we have taken detailed family history and values were also correlated with hematological parameters and in anemic cases HPLC was repeated after treatment of anemia. On HPLC Hb A2 is falsely increased by the presence of HbS adducts, in such cases family screening was useful [11].

Conclusion

The regions with high frequency of beta-thalassemia HPLC are mandatory for screening. However areas with high frequency HbS and laboratories with limited resources can use solubility and Hb electrophoresis as simple and cost effective tool for diagnosis of hemoglobinopathies. However combine approach along is more appropriate for screening and diagnosis of hemoglobinopathies.

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