International Journal of Research in Medical Sciences
Bhokare SB et al. Int J Res Med Sci. 2016 Dec;4(12):5269-5276
www.msjonline.org
pISSN 2320-6071 | eISSN 2320-6012

DOI: http://dx.doi.org/10.18203/2320-6012.ijrms20164193

Original Research Article

Spectrum of hemoglobinopathies by high performance liquid chromatography with special reference to role of HbA2 levels at tertiary care centre

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Received: 25 September 2016
Accepted: 24 October 2016

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ABSTRACT

Background: The inherited disorders of blood include hemoglobinopathies as one of the major public health problems in India. This study indicates type of hemoglobinopathies in a tertiary care hospital over period of 2 years and 3 months.
Methods: total of 500 suspected cases of haemolytic anaemia were studied during the period of July 2013 to Oct 2015 based on Complete Blood Count, Red cell indices and Peripheral blood smear examination. Sickling test, test for Hb quantitation by using cation exchange HPLC was done in all cases.
Results: Out of all 500 cases of anaemia, 313 cases (62.6%) were confirmed to nonhemolytic anaemia whereas 187 cases (37.4%) had shown abnormal haemoglobin pattern on electrophoresis. Out of these 187 cases, 87 (46.52%) were Males and 100 (53.48%) were females. Most common haemoglobinopathy observed was Sickle cell trait 94 (18.8%) followed by beta-Thalassaemia Trait 33 (17.64%), sickle cell-thalassemia trait 27 (14.43%), beta thalassemia major 18 (9.62%) and 1 case of HbE thalassemia trait. The onset of disease was most prominent in Neonatal to paediatric age group (0-10 years) followed by reproductive age group (21-30 years). Few cases in old age were detected.
Conclusions: Study provides data on the spectrum & pattern of Hemoglobinopathies in a tertiary care centre and importance of HbA2 levels in diagnosis of hemoglobinopathies and cases falling in borderline HbA2 levels. Screening of all anaemic patients should be done for Hemoglobinopathies and proper Genetic counselling must be given to all cases to prevent incidence of cases in future generation.

Keywords: Hemoglobinopathies, HPLC, Thalassaemia

INTRODUCTION

Sickle cell anemia and Thalassemia are the major health problems in our country.[1] Thalassemia are prevalent amongst all population groups irrespective of caste, religion and creed. However, sickle cell disorder is mostly confined to socio-economic groups like scheduled tribes, scheduled caste, and nomadic tribes especially residing in rural area.[1,2] Sickle cell gene is widely spread in the district of Eastern Maharashtra, North Maharashtra and some parts of Marathwada region.[2-4] Laboratory Diagnosis of Hemoglobin disorders is required for confirmation of provisional Diagnosis of significant sickling disorders and beta thalassemia major, explain hematological abnormalities and permit genetic counselling of prospective parents. Present study is aimed at speculating the spectrum of hemoglobinopathies and significance of role of HbA2 levels in diagnosis of

hemoglobinopathies and thalassemias. The HbA2 levels are important in diagnosis of β-thalassemia trait, sickle-β thalassemia compound heterozygosity, Hb E variants. Also we studied to borderline cases of HbA2 levels and other causes for reduced HbA2 levels which require further family studies and other supporting investigations.

METHODS

A total of 500 clinically and haematologically suspected cases of haemolytic anaemia during the period of July 2013 to October 2015 were selected and various investigations were performed. Two ml of Intravenous Blood sample was collected from all cases after obtaining informed consent using Ethylene Diamine Tetra acetic Acid (EDTA) as anticoagulant. All the patients of anemia (OPD and Indoor) showing sickling test positive, patients with thalassaemic blood indices on coulter (MCV<80, MCH<27), patients with hepatosplenomegaly and patients referred from Peripheral Health Centers, Rural Health Centers were used as inclusion criteria. Patient received blood transfusions in last three months were excluded from study. Clinical findings were correlated with all other investigations.

Table 1: Analysate identification windows.[6]

Retention time (minutes)	Band (minutes)	Window (minutes)	Range
F	1.15	0.15	1.00-1.30
P2	1.45	0.15	1.30-1.60
P3	1.75	0.15	1.60-1.90
A0	2.60	0.40	2.20-3.30
A2	3.83	0.15	3.68-3.98
D-window	4.05	0.15	3.98-4.12
S-window	4.27	0.15	4.12-4.42
C-window	5.03	0.15	4.88-5.18

Radiological investigations like USG abdomen, X-ray chest and X-ray hip joints and other specific investigations were done as and when required. Hematological profile of cases was done, which included PS, CBC including RBC Indices, Reticulocyte count, sickling test and if required, bone marrow was done. Samples were run on HPLC machine Bio-Rad variant-II and hemoglobin graph was obtained and diagnosis was made based upon values of different hemoglobin fractions and retention times (Table 1).[5]

Special investigations such as iron studies including serum iron, serum ferritin and total iron binding capacity were performed on the sample obtained from the patients to confirm cases of iron deficiency anemia and in further studies of borderline HbA2 levels cases.[7]

RESULTS

313 cases (90.39%) out of all 500 cases detected to have nonhaemolytic anaemia whereas 187 (9.60%) cases had shown abnormal Haemoglobin pattern. All 500 cases of clinically suspected hemolytic anaemias were included in the study.

They were further evaluated by hematological investigations and high performance liquid chromatography (HPLC) for evidence of haemolytic anaemia at Department of Pathology at tertiary care center. So, after complete haematological work up, HPLC, cases were classified as follows (Table 2).

Table 2: Classification of hemoglobin pattern.

Group	Diagnosis	No. of cases
A	Sickle cell trait (SCT)	94 (18.8%)
B	Sickle cell disease (SCD)	14 (2.8%)
C	Sickle cell - beta thalassemia (SBT)	27 (5.4%)
D	Beta thalassemia trait (BTT)	33 (6.6%)
E	Beta thalassemia major (BTM)	18 (3.6%)
F	Hb-E Trait	1 (0.2%)
G	Normal pattern	313 (62.6%)
Total cases		500

Table 3: Age wise distribution of cases among all the groups (n=187).

Age (Years)	Gr-A (n=94)	Gr-B (n=14)	Gr-C (n=27)	Gr-D (n=33)	Gr-E (n=18)	Gr-F (n=1)	Total
0 to 10	24	2	6	4	18	0	54
11 to 20	20	4	10	6	0	0	40
21 to 30	30	4	10	14	0	1	59
31 to 40	12	4	1	5	0	0	22
41 to 50	4	0	0	4	0	0	8
51 to 60	3	0	0	0	0	0	3
61 to 70	1	0	0	0	0	0	1

Maximum no of cases of sickle cell trait, sickle beta thalassemia and sickle cell disease were found in the pediatric and reproductive age group (11-30 years).most of the cases of beta thalassemia trait were found in the 11-20 years of age group. All cases of thalassemia major were found in the early paediatric age group (Table 3).

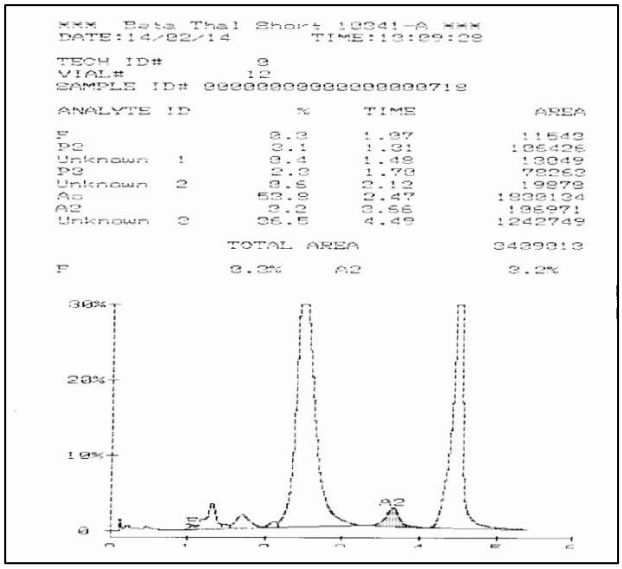

Figure 1: Sickle cell trait.

Out of all the 500 cases 313 cases (90.39%) were confirmed to have nonhaemolytic anaemia whereas 187 (9.60%) cases had shown abnormal haemoglobin pattern on HPLC indicating haemolytic anaemia. These 187 cases consisted of 87 males (59.18%) and 100 females (40.81%). Thus having female preponderance (Table 4). Buddha (58.22%) was the most common ethnic background among all the groups followed by Muslims (16.57%) then Banjara (10.16%).The other caste observed were Maratha (9.62%), Bhill (3.20%), Mang (1.06%) and Mali (1.06%) (Table 5).

Table 4: Sex-wise distribution among all groups.

Group	Male	Female	Total
A	39	55	94
B	10	4	14
C	14	13	27
D	14	19	33
E	10	8	18
F	0	1	1

The reason for increased incidence among specific ethnic groups may be due to illiteracy and consanguineous marriage among communities.

Table 5: Caste wise distribution of cases in present study.

Caste	Group A	Group B	Group C	Group D	Group E	Group F	Total	%
Buddha	58	3	18	22	8	0	109	58.22
Muslim	12	3	3	9	4	0	31	16.57
Banjara	11	3	2	0	3	0	19	10.16
Mang	1	0	1	0	0	0	2	1.06
Mali	2	0	0	0	0	0	2	1.06
Bhil	5	0	1	0	0	0	6	3.20
Maratha	5	5	2	2	3	1	18	9.62

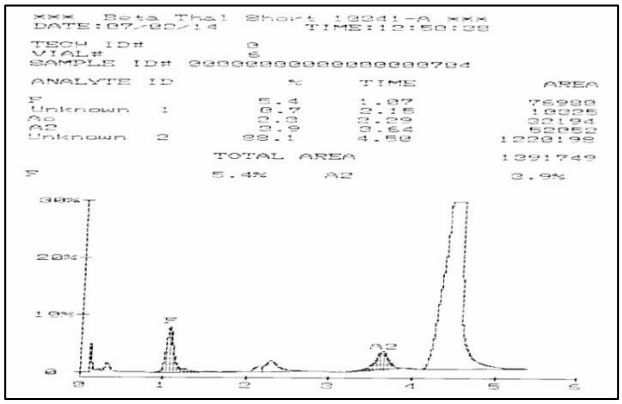

Figure 2: sickle cell disease.

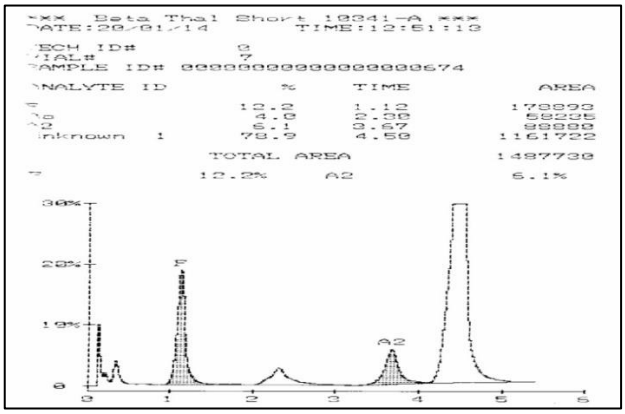

Figure 3: Sickle / Beta Thal.

Clinical presentation

Pallor was most common symptom in all groups but maximum percentage were found in the sickle-β-thalassemia heterozygous state 25 (92.59%) followed byβ-thalassemia major 18 (88.88%), β-thalassemia trait 27 (81.81%), then sickle cell disease 11(78.57%) and sickle cell trait 25 (26.59%) and one case of HbE trait also presented with pallor.

Jaundice was second most common symptom overall β-thalassemia major 13 (72.22%) followed by β-thalassemia trait 18 (54.54%) sickle-β-thalassemia heterozygous state 12 (44.44%), sickle cell disease 3 (21.42%) and sickle cell trait 13 (13.82%). Joint pain was common symptom in sickle-β-thalassemia heterozygous state 16 (59.25%) and sickle cell disease 8 (57.14%) and it was less common in β-thalassemia major 5 (27.77%) and β-thalassemia trait 3(9.09%). Splenomegaly was prominent in β-thalassemia major 15 (83.33%) and sickle-β-thalassemia heterozygous state15 (55.55%).

Splenomegaly was comparatively lower in β-thalassemia trait 5 (15.15%) and sickle cell trait 4(4.25%) (Table 6).

Table 6: Clinical presentation of index cases among all groups.

Clinical features	Pallor	Jaundice	Joint pain	Splenomegaly
Group A (n=94)	25 (26.59%)	13 (13.82%)	18 (19.14%)	4 (4.25%)
Group B (n=14)	11 (78.57%)	3 (21.42%)	8 (57.14%)	2 (14.28%)
Group C (n=27)	25 (92.59%)	12 (44.44%)	16 (59.25%)	15 (55.55%)
Group D (n=33)	27 (81.81%)	18 (54.54%)	3 (9.09%)	5 (15.15%)
Group E (n=18)	16 (88.88%)	13 (72.22%)	5 (27.77%)	15 (83.33%)
Group F (n=1)	1 (100%)	0 (0%)	0 (0%)	0 (0%)
Total	105	59	50	41
% out of 187 cases	56.15	31.55	26.74	21.90

Mean values of hematological parameters is shown in Table 7. Mean values of hemoglobin fractions in all groups are shown in Table 8. Patients with normal hemoglobin pattern were further classified into three groups based upon HbA2 levels as normal (2.5-3.5%), borderline (3.5-3.9%) and reduced HbA2 levels (less than 2.5%). Some patients from borderline group and reduced HbA2 levels with abnormal blood indices were further investigated to rule out other important clinical conditions (Table 9).

Table 7: Mean haematological parameters.

	Group A	Group B	Group C	Group D	Group E	Group F
Hb (g/dl)	9.06±1.87	6.64±1.16	7.32±1.84	7.8±1.48	5.05±1.65	9
MCV (fl)	67.83±5.40	70.14±5.62	70.33±8.85	64.48±8.56	63.19±18.16	78
MCH (pg)	26.88±4.07	24.42±4.25	24.22±4.19	22.4±5.14	24.15±4.05	25
MCHC (g/dl)	31.32±3.89	29.57±2.84	29.34±3.23	27.6±7.06	28.94±2.62	30

Table 8: Mean Values of hemoglobin fractions of Hb disorders on HPLC.

	Mean values of all hemoglobin fractions on HPLC (Mean± SD)			
Group	HbA1	HbF	HbS	HbA2
A (SCT)	54.64±5.50	0.98±1.34	35.42±3.91	3.44±0.50
B (SCD)	8.62±10.14	13.89±9.43	73.3±13.62	3.33±0.46
C (SBT)	5.66±5.11	23.17±9.25	64.64±10.42	5.51±0.90
D (BTT)	84.51±1.47	1.18±1.62	0.0	5.27±0.55
E (BTM)	5.81±2.15	94.94±5.04	0.0	4.06±1.62
F (HBET)	64.1	0.5	0.0	27.8*

*The value 27.8% obtained in Group F is with retention time 3.80, so it was considered as HbE.

Table 9: Classification of normal hemoglobin pattern depending upon HbA2 levels (n=313).

	HbA2 levels (<2.5%)	HbA2 levels between (2.5% to 3.5%)	HbA2 levels (3.5% to 3.9%)
No of patients	73 (23.32%)	232 (74.12%)	8 (2.56%)
Lowest HbA2 levels	1.4	2.5	3.6
Highest HbA2 levels	2.4	3.5	3.7

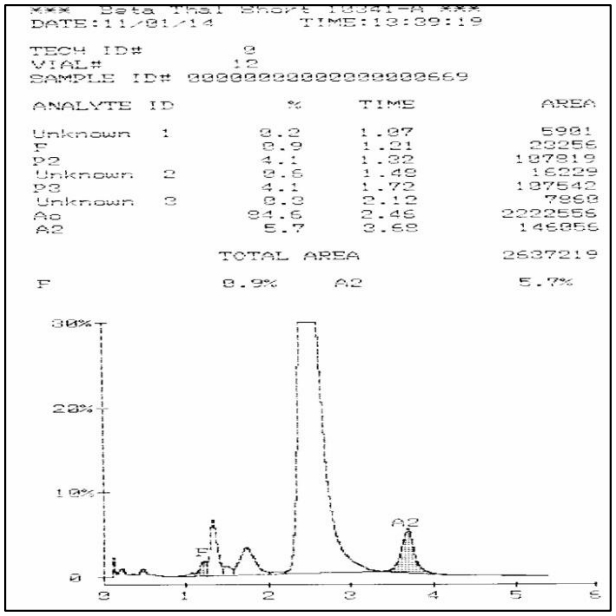

Figure 4: Beta thal trait.

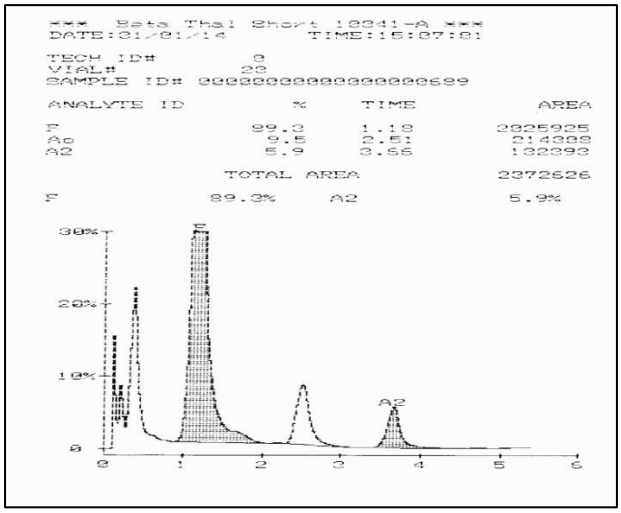

Figure 5: Beta-thalassaemia-major.

Most of the patients (232) were having normal range of HbA2 levels of 2.5 to 3.5%. Eight patients were having borderline HbA2 levels between 3.5 to 3.9%.These cases needed further evaluation to rule out other causes of raised HbA2 such as β-thalassemia with iron deficiency, Megaloblastic anemia, hyperthyroidism, α gene triplications and antiretroviral therapy. Lowest HbA2 levels in this group was 3.6% and highest of 3.7%.

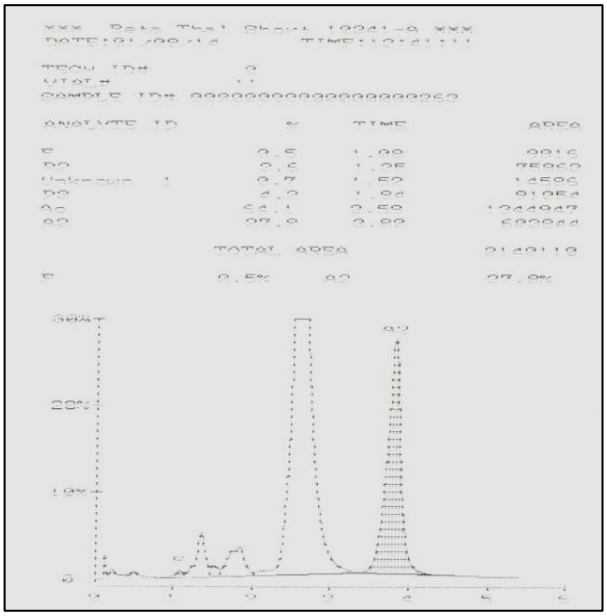

Figure 6: Hb E-trait.

Many cases (73) were showing reduced HbA2 levels less than 2.5% with altered blood indices. Lowest HbA2 levels in this group was 1.4% and highest was 2.4%.These cases also needed further investigations to rule out decreased cause of HbA2 levels such as severe iron deficiency or δ/α gene defects or α-thalassemia trait defects. Remaining 61 cases with reduced HbA2 levels but with normal blood indices. So these patients were labeled as normal and not investigated further.

Out of 7 cases with borderline levels of HbA2 levels 4 cases were provisionally diagnosed as Megaloblastic anemia, 2 cases as β-thalassemia trait with iron deficiency anemia and one case as hyperthyroidism based upon further investigation done on these patients.

DISCUSSION

In present study, Sickle cell trait was most common (18.8%) Hemoglobinopathy detected. Next common Hemoglobinopathy was Beta-Thalassaemia trait (6.6%), followed by Sickle-Beta thalassaemia (5.4%), Beta-Thalassaemia Major (3.6%), Sickle cell anaemia (2.8%), Hb-E trait (1 case) rare Hemoglobinopathy detected. From many previously described studies, Beta-Thalassemia trait is most common hemoglobinopathy in India.[10-12] In present study, Sickle cell trait being most common Hemoglobinopathy detected as present study

was hospital based, not community based and during period of study, large numbers of solubility positive samples from peripheral health centers were received. In the present study Occurrence of Hemoglobinopathies was highest in Buddhas (58.22%), followed by Muslim (16.57%) and Banjaras (10.16%). Other studies also reported maximum report of cases of sickle cell disorder in mahar caste (11.08%) by Bhaskar et al and Ambekar S.S. et al reported maximum cases of beta thalassaemia amongst Navbuddhas.[8,9] In the present study splenomegaly was found most common in the patients of β- thalassemia major (83.33%) followed by sickle-Beta thalassemia (15 cases), sickle cell Anemia (2) and sickle cell trait (4).

In this study we considered HbS value of less than 40% for diagnosis of sickle cell trait and HbS value of more than 50%, HbA2 less than 4% and mildly raised HbF levels for diagnosis of Sickle cell disease. Sickle cell disease and sickle cell trait show high level of HbA2 levels maybe because of HbS adducts (carbamylated and glycated) which has high affinity for coeluting with HbA2. Vasaikar M et al and Colah R et al stated that HbA2 was found to be significantly higher in HbS trait cases than the range for HbA2 in the normal.[10,13]

In this study we considered microcytic hypochromic blood picture with HbA2 levels between 4-9% for diagnosis of β-Thalassemia trait. Biruah MK et al also stated that HbA2 levels of 4-9% are diagnostic of BTT in an asymptomatic individual with no or mild anemia. HbA2 ranged from 4.0% to 8.5% with a mean of 5.3%.[14] The HbE elute into the HbA2 window but differentiated upon the retention time and Hb fraction %.In present study we found one case of HbE trait with HbE value of 27.8%. Mondal SK et al in their study found HbA2, HbE and Hb Lepore to elute in A2 window (retention time ±3.3-3.9 min).[14,15] Other than the importance role of HbA2 in the patients with β-thalassemia trait, sickle β thalassemia and HbE variants, there are also other causes which influence on HbA2 levels and may cause underdiagnosis or misdiagnosis. We got 7 cases which showed borderline raised HbA2 levels (3.5-3.9%) which were investigated further. Also we got 73 cases which showed reduced HbA2 levels less than 2.5%, out of which 12 cases which were having altered blood indices and severely reduced HbA2 levels less than 2% were investigated further.

In present study five most commonly affecting factors on HbA2 levels are discussed as follows

Factors causing borderline raised HbA2 levels:

- Megaloblastic anemia,
- Hyperthyroidism,
- Iron deficiency with β-thalassemia trait, And Factors causing reduced HbA2 levels:
- Iron deficiency in normal individuals
- Presence of α/δ gene defects and α-thalassemia trait.

In present study we found 4 cases (Table 10), with borderline raised HbA2 levels and raised MCV >110 fl, on peripheral smear and bone marrow findings confirmed megaloblastic anemia. Rao S et al in their study stated that 19 patients with megaloblastic anaemia showed HbA2 levels (3.32±0.56%) which was significantly higher (P<0.001) than the HbA2 levels (2.89 ± 0.37%) of normal cases with MCV<110fl.[13]

Table 10: Patients with normal hemoglobin pattern and borderline HbA2 levels with further investigations (n=7).

Total cases	4	1	2
Hb (gm%)	5.5	8	5
MCV(fl)	118	96	60
MCH (pg)	39	28	24
MCHC(gm/dl)	33.7	33	28
HbA1 (%)	83.1	85.6	82.2
HbF (%)	3.2	1.5	1.5
HbA2 (%)	3.9	3.7	3.7
Serum ferritin(µg/dl)	22	20	10
Serum iron (µg/dl)	35	35	20
TIBC(µg/dl)	320	280	400
PS Examination	Macrocytic picture	NCNC	Micro, hypo
Bone marrow examination	Megaloblastic anemia	Normoblastic	Micronormoblastic
Clinical history	Pallor, weakness	Palpitation, thyroid swelling	Weakness, palpitation
Family studies	Not significant	Not significant	Mother is β-thal trait
Provisional diagnosis	Megaloblastic anemia	hyperthyroidism	β-thal trait with iron deficiency anemia

Vasaikar M et al also stated that HbA2 value upto and above 4% can be observed in cases of megaloblastic anemia.[10] In present study one studied one case (Table 10), which showed HbA2 level of 3.9% and raised MCV. On further investigation peripheral smear and bone marrow examination were normal. But clinically the patient was having symptoms of hyperthyroidism such as palpitation, thyroid swelling. So the patient was suspected for hyperthyroidism and was referred for thyroid function tests. Patient was lost to follow-up. Kendall AG et al stated that Hb A2 was significantly elevated (mean± SD, 3.3±0.5%: normal, 2.5±0.3%: p less than 0.001) in 28 hyperthyroid patients prior to antithyroid therapy.[16]

Kuhn GM et al studied 128 women for effect of hyperthyroidism on HbA2 levels. The mean (±SEM) HbA2 level in was higher (P less than 0.001) in hyperthyroid patients without treatment (3.21±0.06%) than in hyperthyroid patients with treatment (2.42±0.09%) and Healthy individuals (2.48±0.04%).[17]

In present study we studied 2 cases (Table 10), in which we got borderline HbA2 levels 3.7% and microcytic, hypochromic picture on peripheral and bone marrow examination. As stated earlier one of the reason for borderline HbA2 levels maybe coexistence of β-thalassemia trait with iron deficiency anemia, So the patients were suspected and parental studies were done. And one of the parent was found to be beta thalassemia carrier. So patients were provisionally diagnosed as beta thalassemia trait with iron deficiency and molecular testing and iron therapy was advised. Patient was lost to follow-up.

A Mosca et al stated that decreased HbA2 levels can be detected in iron depletion, possibly due to the preferential binding of β to αchains, rather than δ chains, or to an inhibition of low iron levels on δ globin synthesis. However, in other cases (patients from the Indian subcontinent) who are iron depleted or frankly iron deficient, HbA2 has sometimes been observed to be reduced to the normal range (Barbara J Bain, personal communication).[18]

Atul shrivastav et al stated that Iron deficiency may lead a low Hb A2 and hence may mask α-thalassemia trait.[11,19] Out of 73 cases with decreased HbA2 levels only 12 cases showed markedly reduced HbA2 levels (1.4-2%) and altered blood indices. Of which 10 cases were suspected for iron deficiency anemia and two cases were suspected for δ/α gene defects or α-thalassemia trait defects. Of these two representative cases are discussed here.

Two cases (Table 11), were having HbA2 levels around 1.8 to 2.0 % with reduced blood indices and normal iron studies. Peripheral smear and clinical history was not significant. So considering hematological findings and family history these patients were suspected for α-thalassemia trait or δ/α gene defects.

Other 10 cases (Table 11), showed moderate to severe anemia and reduced blood indices and decreased HbA2 levels between 1.4-2.0%.

Table 11: Patients with normal hemoglobin pattern and reduced HbA2 levels with further investigations (n=12).

Total cases	2	10
Hb (gm%)	4.8	5.2
MCV(fl)	60	54
MCH(pg)	22	20
MCHC (gm/dl)	29	24
HbA1 (%)	85	87.7
HbF (%)	1.6	0.3
HbA2 (%)	2.0	1.4
Serum ferritin (µg/dl)	25	12
TIBC(µg/dl)	300	410
Serum iron(µg/dl)	32	18
PS examination	Micro, hypo	Micro, hypo
Bone marrow examination	Normoblastic	Micronormoblastic
Clinical history	Not significant	Weakness, palpitation
Family studies	Not significant	Not significant
Provisional diagnosis	α-thalassemia trait/ delta-alpha gene defects	Iron deficiency anemia

Further investigations showed microcytic hypochromic picture on PS and BM. Iron studies also showed decreased serum iron, serum ferritin and increased total iron binding capacity. So these patients were diagnosed as iron deficiency anemia. A Mosca et al stated that reduced HbA2 levels can be detected in the presence of α-thalassaemia, probably again due to preferential binding of the scarce a chains with the β rather than with the δ counterparts. This is especially clearly seen in HbH disease, where HbA2 can drop to less than 1%.[18] G B Tan et al studied 25 cases of α-thalassemia trait which showed HbA2 levels 1.4-2.6% and mean of 2.0%.[20]

CONCLUSION

HPLC is Rapid, automated, accurate, and reliable method for quantification of hemoglobin variants in screening of large population. Role of HbA2 levels is most important in diagnosis of beta thalassemia trait, sickle beta thalassemia and Hb variants eluting into the HbA2 retention window. Cases with borderline HbA2 levels should be further investigated such as careful clinical examination, iron studies, parental studies and genetic studies as these patients may get underdiagnosed or

missed (coexistent beta thalassemia with Iron deficiency anemia, megaloblastic anemia, and hyperthyroidism).

Reduced HbA2 levels (1.5-2.0%) can be found in severe iron deficiency, α-thalassemia trait and δ/α gene defects. These cases should be further investigated such as Iron studies, parental studies and molecular studies should be performed in cases wherever necessary.

Funding: No funding sources
Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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Cite this article as: Bhokare SB, Phulgirkar PP, Joshi AR, Bindu RS. Spectrum of hemoglobinopathies by high performance liquid chromatography with special reference to role of HbA2 levels at tertiary care centre. Int J Res Med Sci 2016;4:5269-76.