



## HPLC based evaluation of Haemoglobinopathies in a tertiary care setting in North Kerala

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### Abstract

**Introduction-** *Inherited Haemoglobin disorders are the most common single gene disorders in the world with the highest prevalence in regions where malaria was endemic. The burden of this disorder is of such a magnitude that it represents a major public health concern in the majority of the regions. The common variants prevalent in India are Hb S, Hb D-, and Hb E. Hb S and Hb E are the common variants prevalent in tribals of India. In Kerala, Hb S is prevalent in tribals of Wayanad and Attapadi, also in a nontribal Chetty community in Wayanad. But the existence of other hemoglobinopathies is still not properly evaluated. The aim of the present study was to analyze laboratory aspects- hematological profile and HPLC findings of the various hemoglobin variants detected from our Laboratory.*

**Materials and methods-** *A total of 4200 cases received from January 2010 to December 2016 which came for HPLC to evaluate anaemia. The tests were performed on HPLC instrument BIO-RAD –D10. The principle of the Instrument is Cation Exchange High-Performance Liquid Chromatography. Complete blood count and the peripheral smear of the samples were done in every case. Hb electrophoresis - both acid and alkali were done in selected cases. Family studies were also done in needed cases whenever the situation demanded it.*

**Results-** *A total of 4200 cases were studied. Of these, in 1258 cases we could detect abnormal hemoglobin on HPLC, Out of the 4200 case, 2395 cases were from the tribal population of Wayanad, Malappuram, and Palakkad. The major abnormality detected were Hb S and Beta –thalassemia syndromes.*

**Conclusion-** *HPLC forms a rapid, accurate and reproducible tool for early detection and management of hemoglobinopathies and variants. Hb S and thalassemias are the common abnormalities found in Northern Kerala.*

### INTRODUCTION

Inherited Haemoglobin disorders are the most common single gene disorders in the world with the highest prevalence in regions where malaria was endemic. The burden of this disorder is of

such a magnitude that it represents a major public health concern in majority of the regions.<sup>1</sup>

Haemoglobinopathies can be quantitative (as in thalassemia syndromes) or qualitative (as variant Hbs). Thalassemia is an autosomal recessive

inherited group of disorders of hemoglobin synthesis where there is absence or reduction of one or more of the globin chains of hemoglobin. The structural variants result from structural variations like the substitution of one or more amino acids in the globin chains of the hemoglobin. There are many hemoglobin variants prevalent in India because of its ethnic diversity .they also have a varied clinical impact. These hemoglobinopathies are usually recessively inherited from the parents, and they lead to severe morbidity and mortality in Indian population. The common variants prevalent in India are Hb S, Hb D-Punjab and Hb E .Hb S and Hb E are the common variants prevalent in tribals of India .<sup>2</sup> In Kerala, Hb S is prevalent in tribals of Wayanad and Attapadi , also in a nontribal Chetty community in Wayanad. But the existence of other hemoglobinopathies is still not properly evaluated.<sup>3</sup>

Cation exchange high-performance liquid chromatography (CE-HPLC) has to lead to a simple rapid and superior method for detection and quantification of these variants, in routine laboratory practice.<sup>4</sup> Detection of asymptomatic carriers and double heterozygous states are the most important advantages of this method. Early, accurate detection of carriers can aid in prevention and management of various hemoglobinopathies. The aim of the present study was to analyze laboratory aspects- hematological profile and HPLC findings of the various hemoglobin variants detected from our Laboratory.

## OBJECTIVES

To assess the spectrum of hemoglobinopathies detected by HPLC during the period from January 2010 to December 2016.

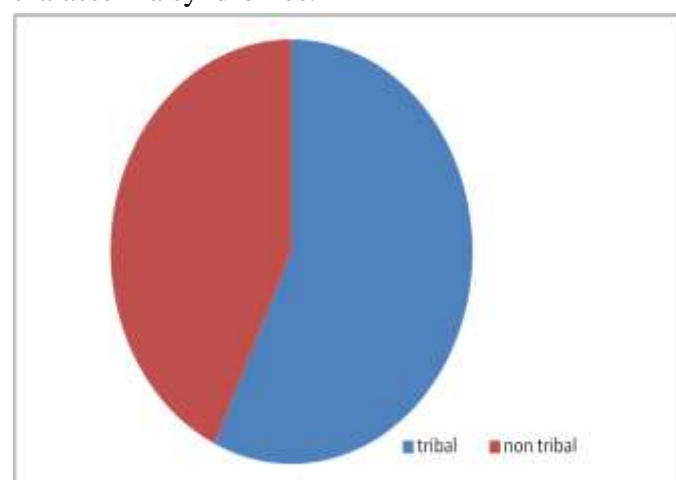
## MATERIALS AND METHODS

A total of 4200 cases received from January 2010 to December 2016 which came for HPLC to evaluate anaemia. The geographical distribution of all the cases predominantly included parts of northern Kerala. The tests were performed on HPLC instrument BIO-RAD –D10. The principle

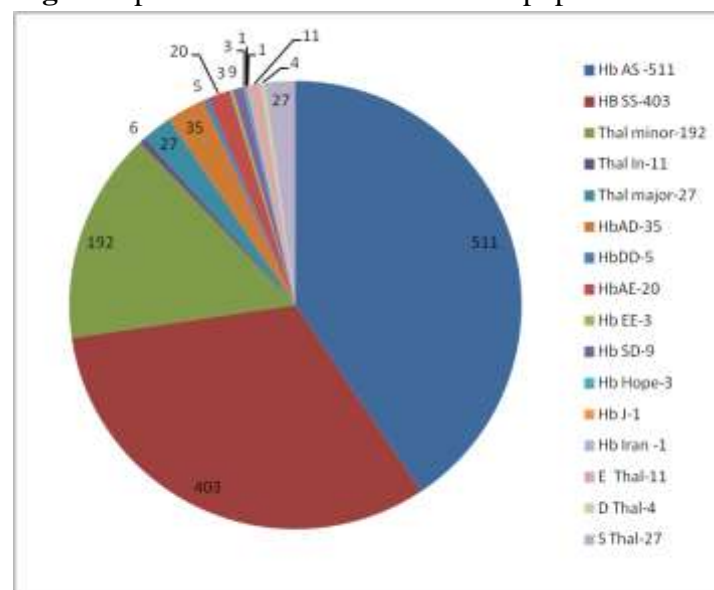
of the Instrument is Cation Exchange High-Performance Liquid Chromatography. Complete blood count and the peripheral smear of the samples were done in every case. Hb electrophoresis - both acid and alkali were done in selected cases. Family studies were also done in needed cases whenever the situation demanded it.

## RESULTS

A total of 4200 cases were studied. Of these, in 1258 cases we could detect abnormal hemoglobin on HPLC (fig.2), Out of the 4200 case, 2395 cases were from the tribal population of Wayanad (93%) , Malappuram and Palakkad. (fig.1). The major abnormality detected were Hb S and Beta – thalassemia syndromes.



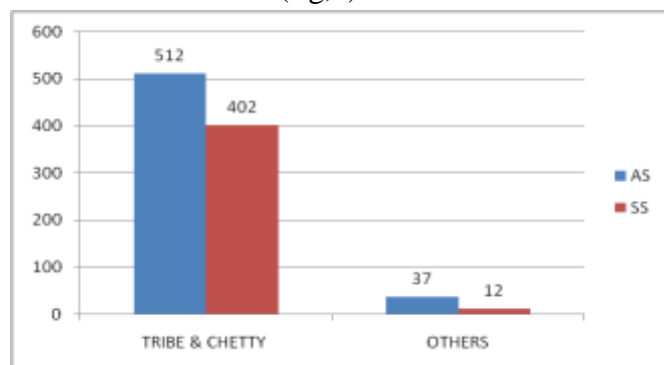
**Fig.1** Proportion of tribal and nontribal population



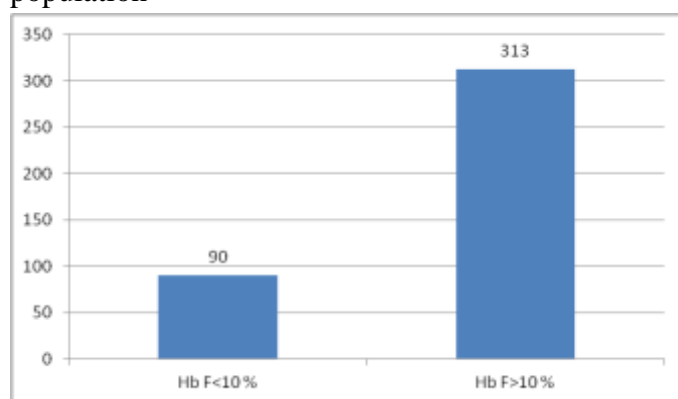
**Fig. 2** Haemoglobinopathies

Hb S is detected as S- window. There were 511 cases of HB AS and 403 cases of HbSS. Majority of the cases were from tribes of Wayanad district.

Out of 914 cases (73%), only 49 cases were from other community (fig,3). Hb SS cases had a strong association with raised Hb F. 313 out of 403 cases had a Hb F of >10% (fig,4).



**Fig.3** Sick cell disease in tribals and chetty population



**Fig,4** Hb F in sickle cell anaemia

A cut-off value of over 4 % Hb A2 was taken for diagnosis of beta thalassemia trait. A total of 192 cases (15%) of beta thalassemia trait were diagnosed. Correlation with peripheral blood findings as microcytic hypochromic anaemia with raised RBC counts was confirmed in the majority of cases.

There were 27(3%) cases of beta thalassemia major and 6 cases of thalassemia intermedia. Cases diagnosed with beta-thalassemia intermedia were clinically symptomatic and had a variable degree of anemia with microcytic hypochromic blood picture. Hb F levels were also raised with variable values in individual cases.

Cases diagnosed with thalassemia had marked anemia, anisopoikilocytosis, microcytic hypochromic blood picture with polychromasia and normoblasts in the peripheral smear.

There were 27 cases of double heterozygosity for sickle- beta thal syndromes and 11 cases E –Beta

thalassemia syndromes and 5 cases of Hb D-beta thalassemia syndromes.

Hb D- Punjab heterozygous constituted 35 (3%) cases whereas homozygous Hb D was only 5 cases. The elution time of Hb D is 3.8 minutes. Out of the 40 cases, 28 cases were from the Muslim population of northern Kerala.

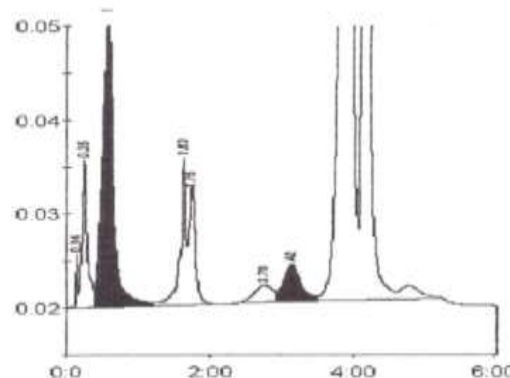
Only one case had isolated Hb F elevation with normal blood counts. The possibility of hereditary persistence of fetal hemoglobin was considered and confirmed by kliehaeur –betke test which showed a pancellular distribution of Hb F.

Hb E variant included 3 cases of Hb E homozygous and 20 cases of Hb E heterozygous. Hb E presents as a raised peak in the A2 region with retention times ranging from 3.68-3.79 minutes. Out of the 23 cases(2%), 10 cases were from outside Kerala – the majority from West Bengal and Orissa.

There was one case of Hb J and 3 cases which raised the suspicion of Hb Hope.

#### Patient report

Bio-Rad DATE: 07/22/2015  
D-10 TIME: 01:42 PM  
S/N: #DB8A197016 Software version: 3.53  
Sample ID: AMAL-29-M  
Injection date: 07/22/2015 01:42 PM  
Injection #: 19 Method: HbA2/F  
Rack #: — Rack position: 5



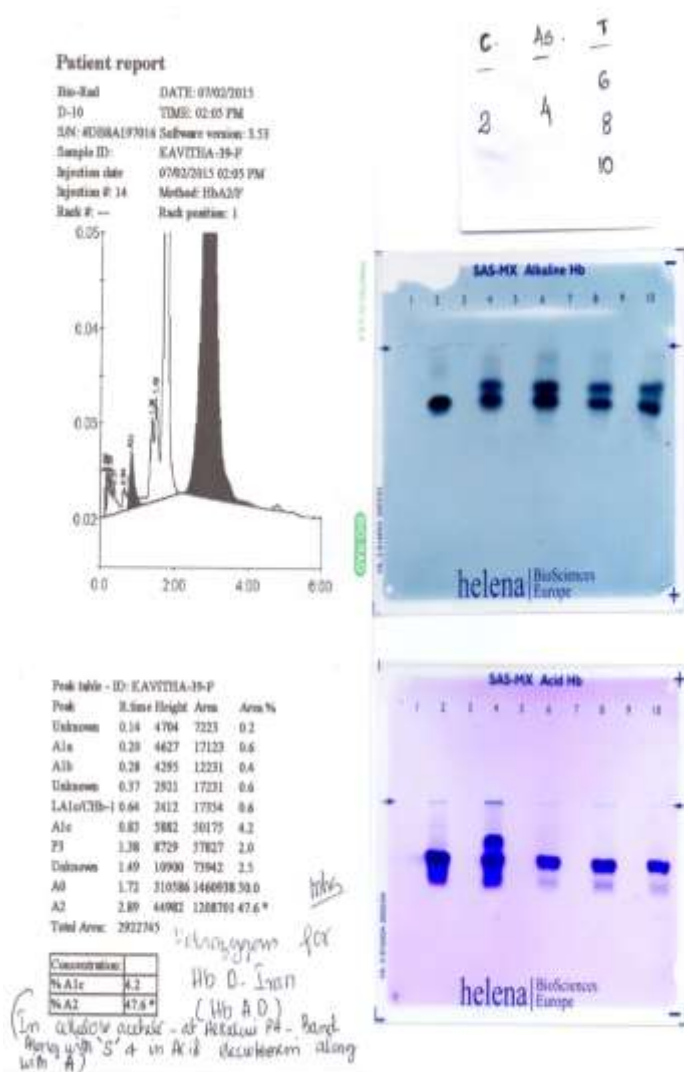
Peak table - ID: AMAL-29-M

Peak	R.time	Height	Area	Area %
Unknown	0.14	5944	9621	0.3
A1a	0.25	15611	79308	2.8
F	0.58	31256	316046	11.3
A0	1.63	15589	81521	2.9
Unknown	1.75	12614	74903	2.7
Unknown	2.76	1841	33173	1.2
A2	3.14	4036	65861	2.7
Unknown	3.90	125691	1202273	43.0
S-Window	4.17	186017	934739	33.4
Total Area:			2797445	

Concentration:	
% F	11.3
% A2	2.7

Haemoglobin SD  
Disease  
mha

**Fig -5** chromatogram of Hb SD Disease



**Fig 6** Heterozygous for Hb D-Iran

## DISCUSSION

The gene frequency of Haemoglobinopathies in India is 4.2%. The commonest among the Haemoglobinopathies is thalassemia. Clinically significant thalassaemic disorder in India is beta-thalassemia while alpha thalassaemia is free from morbidity. Hb S, HbD, and Hb E are the other prevalent Hb Variants.

In Kerala also the most prevalent Haemoglobinopathy is thalassemia syndromes. But the gene frequency of Hb S is high in tribal communities of Kerala .1-4 In Kerala, 1.5% of the population is represented by tribes, of which 36.5% resides in Wayanad. A significant population our cases are tribes from Wayanad district. Since every tribal patient from Wayanad, coming to the hospital was sent to us for screening of Hb S, we had high percentage Hb S. <sup>5</sup>

Five distinct haplotypes of Hb S gene have been described in different geographical areas. Benin, Senegal, Bantu, and Cameroon are the African haplotypes while the 5th one is the Saudi Arabia / Indian haplotype. <sup>6</sup> This haplotype is associated with higher levels of Hb F which ameliorates the clinical severity. If associated with  $\alpha$  - thalassemia mutations also help in making the clinical severity of sickle cell disease milder. Our patients also had a high percentage of Hb F. But the association of  $\alpha$  - thalassemia has to be evaluated further.

Beta thalassemia trait formed the second largest subgroup of abnormal hemoglobin (15%). The characteristic hematological findings in a typical case of beta thal trait include mild anaemia, microcytosis with raised RBC counts. Hemoglobin may be normal or slightly reduced. The mutations common in an India are IVS1-5(G-C), 619 bp deletion, IVS 1-1(G-T), CD8/9(+G), CD41/42(-CTTT), CD15 (G-A), CD30 (G-C). 619bp deletion is associated with high Hb A2 values. Conditions with borderline Hb A2 need careful interpretation. Iron deficiency may lead a low Hb A2 and hence may mask minor but in contrast, B12/folate deficiency may lead to slightly high Hb A2 values leading to a false diagnosis of beta thalassemia minor. Proper evaluation of indices with iron studies will be useful in such cases. Co-inheritance of delta thalassemia may also lead to borderline A2 levels. Genetic studies should be advised in such cases for confirmation. <sup>7</sup>

Thalassemia major and intermedia constituted approximately 3 % of cases. 17 out of 27 cases of thalassemia major presented in the first decade of life, rest in the second decade. They had severe anaemia and severe microcytosis with evidence of hemolysis. All patients were transfusion dependent.

Hb D-Punjab is a sub-variant of Hb D common in Punjab region of India and Pakistan. They have a normal phenotypic presentation. There is a mutation in the beta chain at b121 Glu @Gln (GAA-CAA).<sup>8</sup> Patients with coexistent Hb D and thalassemia usually have milder disease. Double heterozygosity of Hb D with Hb S leads



moderately to severe clinically symptomatic disease. We had around 40 cases (3%) of Hb D disease and also 9 cases (1%) of deadly combination of Hb S & D in 5 families.

Hb E constituted 23 cases (2%) of our cases with only 3 homozygous states and few combined with thalassemia trait. 10 cases out of these are from outside Kerala- from West Bengal and Orissa since Kerala having manual laborers from these areas. Hb E usually elute along with Hb A2. High values of Hb A2 above 10 % raise the suspicion Hb E. Acid and alkali electrophoresis may help to differentiate it from Hb D – Iran which also elutes along with Hb A2 & Hb E.

Clinical features include homozygous cases are anemia, microcytic hypochromic RBCs with target cells. Hb E Heterozygous individuals are clinically asymptomatic. Double heterozygous HbE -beta thalassemia trait is important and symptoms resemble those of thalassemia major.<sup>9</sup> Hb D-Iran On HPLC, in the A2 window similar to Hb E. Acid and alkali electrophoresis, may be useful in diagnosis. Hb E move along with Hb A2 in both acid and alkali electrophoresis, but Hb D will be moving along with Hb S in alkali electrophoresis. We had only one case of Hb D-Iran.<sup>10</sup>

Hb J presents as an elevated P3 peak on HPLC. Hb J is usually asymptomatic or patients may present with mild anemia. Hemoglobin electrophoresis at alkaline pH shows a fast-moving band ahead of Hb A. Hb J Meerut is an alpha chain variant with a retention time of 1.88 minutes.<sup>11</sup>

We also had 3 cases suspicious of Hb Hope which eluted along with Hb A1c .2 cases were diabetic patients who came for regular follow up. But since this Hb is eluting along with Hb A1c it posed problems in reporting Hb A1c values.<sup>12</sup>

A limitation of cation exchange – HPLC technique is that possibility of alpha thalassemia, normal A2 beta thalassemia or other hemoglobinopathies that elute with similar retention values on HPLC cannot be ruled out.

To conclude, HPLC forms a rapid, accurate and reproducible tool for early detection and

management of hemoglobinopathies and variants. Hb S and thalassemias are the common abnormalities found in northern Kerala. Detection of other variants becomes important due to complex interactions in cases with double heterozygous and homozygous states, which may lead to severe hematological abnormalities. Findings must be supplemented by hemogram findings, family/sibling studies, hemoglobin electrophoresis, other confirmatory techniques and molecular studies based on HPLC findings.

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