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RESEARCH ARTICLE

PATHOLOGY

SPECTRUM OF HEMOGLOBINOPATHIES DIAGNOSED BY HPLC IN HIGH PREVALENCE AREA OF NORTH MAHARASHTRA.

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ABSTRACT

The present study aims at evaluating importance of Cation exchange HPLC in high prevalence area in North Maharashtra 4394, samples analyzed in the BIO-RAD variant HPLC system. The retention times, proportion of Hb% and the characteristic of peak for all the Hb fractions were recorded. Screening test and electrophoresis at alkaline PH were done in all cases. Abnormal hemoglobin fractions on HPLC were seen in 2726 cases. HbSA was the predominant in 2253 cases, 270 cases ofHbSS, 183 cases of beta Thalassemia trait, 3 cases of Beta homozygous Thalassemia, 10 cases of Double heterozygous HbS-beta Thalassemia, 3 cases of Hb D trait, 3 cases of Hb E trait and 1 case of Double heterozygous HbE-beta Thalassemia. HPLC was found to be simple, rapid and reliable method. HPLC should be used as an additional method for identification of hemoglobin variant where the work load is more and automation is necessary.

KEY WORDS

High Performance liquid chromatography (HPLC), Sickle cell trait (HbSA), Sickle cell disease (HbSS), Hemoglobin E (HbE), Hemoglobin D (HbD).

INTRODUCTION

Sickle cell anemia and Thalassemia are the major health problems in our country [1].Thalassemia is prevalent amongst all population groups irrespective of caste, religion & 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].

Sickle cell disorder & state of Maharashtra-

According to 2001 census, total population of Maharashtra is 96,878,627. The population of the state is second in the country, of this 8.9% are scheduled tribes, and 87% resides in rural area. Expected sickle cell carriers are 10%, expected suffers 0.5%.

From the available data, it is found that sickle cell gene is widely spread in the district of Eastern Maharashtra, North Maharashtra[2] and some parts of Marathwada region[3]. It is also estimated that district with more than 5000 cases of sickle cell anemia are seen in Nandurbar,Gadchiorli,Chandrapur, Nagpur,Yewatmal, Bhandara district of Maharashtra. Highest prevalence is seen in Nandurbar and Gadchiroli district 20%[4].

The disease is incurable and hence patients are not only physically affected but mentally too. Due to presence of sickle cell anemia patient the whole family is affected.

The Maharashtra state government is aware of the grave problem and so has launched sickle cell prevention and control program since 2008.

The identification of abnormal hemoglobin is often presumptive based on electrophoresis mobility. This presumptive identification should be based on a minimum of two techniques based on different principles[5],Thinking of volume of workload, ease of handling, reproducibility, and expertise. The tertiary units of sickle cell prevention and control programme are situated in Medical colleges which are near the high prevalence area and they are provided with fully automated systems such as Cation exchange HPLC.

Cation exchange HPLC offers a tool for early, accurate detection of Hemoglobinopathies there by aiding in prevention and management of hemoglobinopathies.

The present study aims at evaluating importance of Cation exchange HPLC in high prevalence area where the workload is more and to see spectrum of hemoglobinopathies besides sickle cell anemia in North Maharashtra.

MATERIALS AND METHODS

This was a prospective study carried out in the tertiary centre of Shri Bhausaheb Hire govt. Medical College Dhule over a period of three years from the year 2008-2010.Our college is situated close to highest prevalence (20%) Nandurbar district. Similarly, along with Dhule district, Nashik&Jalgaon district which too is close to Dhule have high prevalence of sickle cell disease.

A total of 24723 populations were screened in three years period. This includes mass screening of OPD patients, ANC patients, Patients admitted in wards of our college hospital along with camps conducted by primary & secondary units situated in Nandurbar, Dhule, Nashik, Jalgaon district. Screening tests i.e. solubility test for sickle cell disorder, Nestroff's test for Thalassemia was done. Patients having positive screening test along with patients having negative screening test but suspicious of having hemoglobinopathies in such patients electrophoresis on cellulose acetate at alkaline ph was done in secondary units. Samples were

collected into tubes containing dipotassium EDTA (vacutainers). The samples were run on cell counter to obtain hemoglobin value & red cell indices. The same sample was used for HPLC.

A total of 4394 samples were analyzed on HPLC. After collection, the samples were stored at 2-8^{o}c and tested within a week of collection. The samples were run on an instrument manufactured by BIO RAD laboratories. The instrument is known as BI0-RAD variant (Beta Thalassemia short program) utilizes the principle of high performance liquid chromatography. An HbA_2F calibrator and two levels of controls (BIO-RAD) were analyzed at the beginning of each run. The total area acceptable was between one to three million[6].

RESULTS

The following tables (1&2) show data of three year period (2008-2010) under the sickle cell prevention and control program.

Table 1

Three years data screened under sickle cell prevention control program.

Total Population screened	**24723**
Total samples run on HPLC	4394
Samples showing normal hemoglobin	1668 (6.7%)
Samples showing abnormal hemoglobin	2726 (11.01%)

Table 2

Abnormal spectrum of hemoglobin detected in three year period

Sickle cell trait	2253 (9.11%)
Sickle cell Disease	270 (1.09%)
Beta heterozygous Thalassemia	183 (0.7%)
Beta homozygous Thalassemia	3
Double heterozygous HbS-beta Thalassemia	10
HbD D trait	3
HbE trait	3
HbE-beta Thalassemia	1
Total	**2726**

Total samples run on HPLC were 4394, out of which 1668 (6.7%) were normal (Chromatogram-1).

As expected sickle cell trait was detected more frequently i.e. 9.11% cases. Their HbS ranged between 30-40% and HbS value was less than HbAvalue (Chromatogram-2). Hb ranged from 10-12gm%. MCV was normal. On electrophoresis AS pattern was seen.

1.09% patients showed increased HbS values more than 70% and normal HbA_2 value and increased HbF value (5-20%), (Chromatogram-3). On electrophoresis, all had band at SDG region and solubility test was positive. They were diagnosed as homozygous sickle cell anemia.

In 10 patient HPLC showed HbS values more than 70% and HbA_2 level ranging from 5-7% along with raised HbF (6-20%),(Chromatogram-6). They showed low MCV, MCH, MCHC, RBC count was increased. They were diagnosed as HbS-beta Thalassemia. On electrophoresis SFA_2 pattern was seen.

183 patients (0.7%) were diagnosed to have to have beta heterozygous Thalassemia based on high level of HbA_2 (>4.0%)(Chromatogram-4).

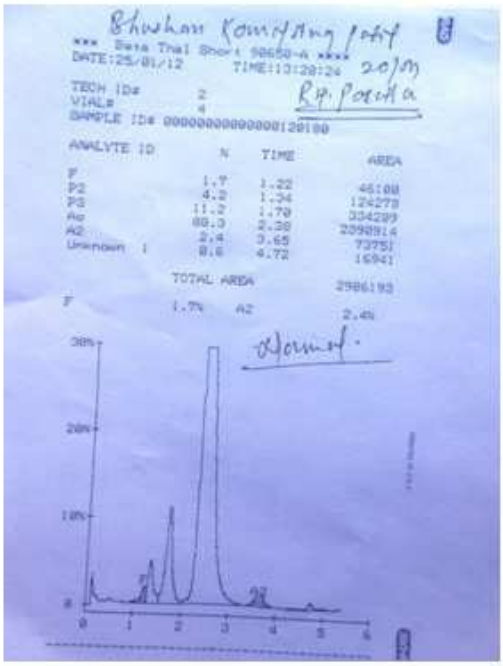
These patients had Hb level less than 9.0 gm% and all showed low MCV<80fl and reduced MCH and MCHC.

Three patient were diagnosed as beta homozygous Thalassemia in whom HbF level was very high above 90%.Hb level was less than 4gm% (Chromatogram-5). MCV and MCH was reduced and patient required frequent blood transfusion.

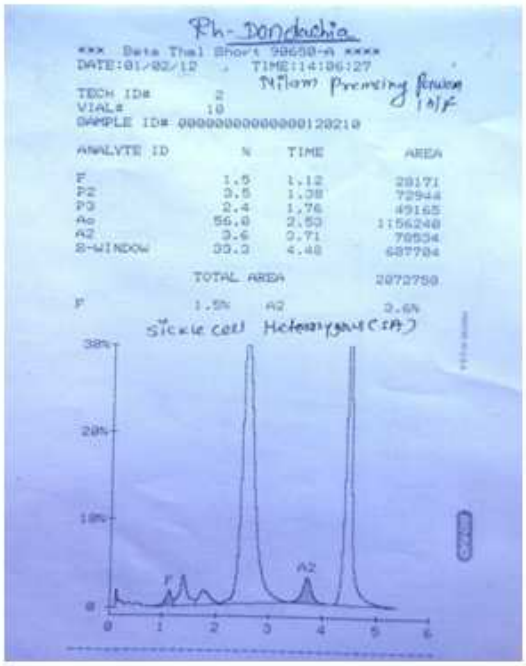
3 patients had Hb band in SDG region on gel electrophoresis and negative solubility test. On HPLC they had unknown peak at D window with retention time of 3.90 to 4.30 min, this was different fromHbS peak of retention time 4.30 to 4.70 minutes. The abnormal hemoglobin constituted between 30-40% of total hemoglobin. They were diagnosed as HbD Punjab heterozygous.

Three patients were diagnosed as HbE heterozygous. They presented as raised peak in the A_2 region with retention time ranging from 3.68-3.79 minutes.HbE heterozygous have an average raised HbE usually less than 40% (Chromatogram-7).

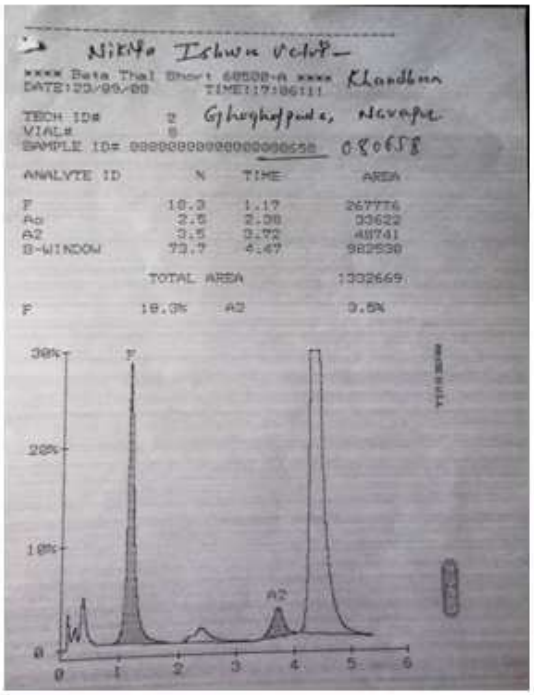
Single case of HbE beta Thalassemia trait double heterozygous was seen. HbE was 48.8% while HbF was 8.2%.The patient had low hemoglobin and MCHC.Herequired blood transfusion. Family history confirmed the case.



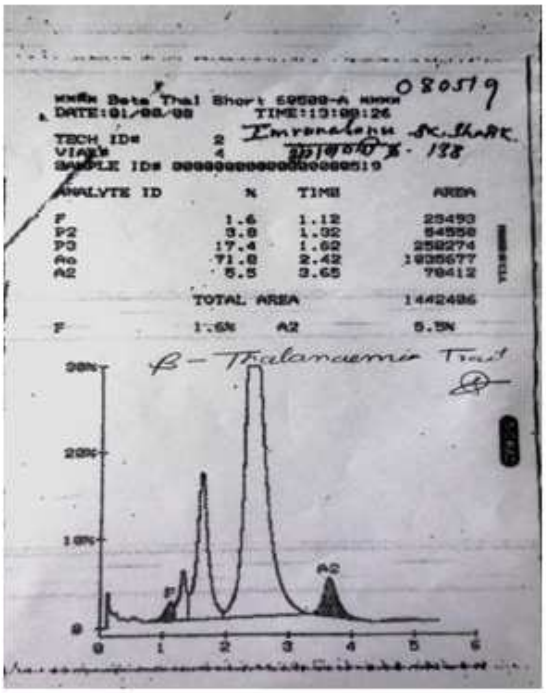
Chromatogram-1
Normal



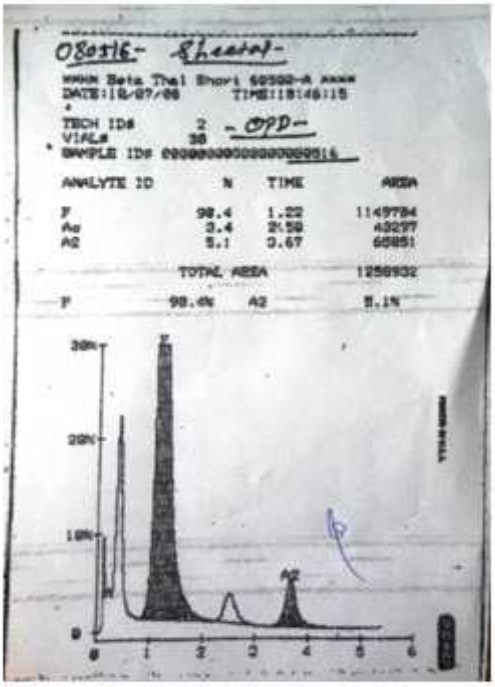
Chromatogram-2.
Sickle cell Heterozygous.



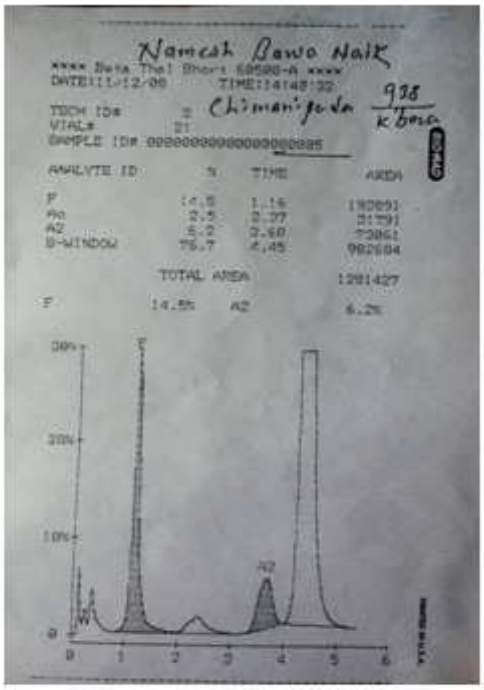
Chromatogram-3.
Sickle cell Homozygous



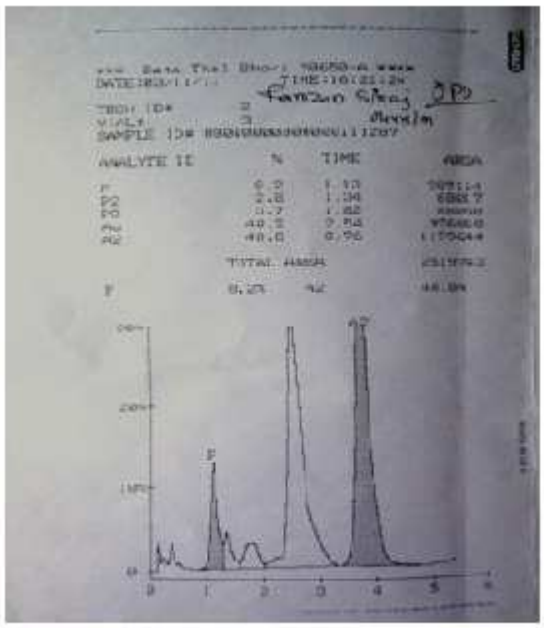
Chromatogram-4.
Beta Heterozygous Thalassemia



Chromatogram-5.
Beta Homozygous Thalassemia
Thalassemia



Chromatogram-6.
Double heterozygous
HbS-beta



Chromatogram-7.
Hemoglobin E (Hb E)

DISCUSSION

Our findings of 9.11% sickle cell trait patients and 1.09% sickle cell disease patients do co-relate with the findings of S L Kate i.e. the overall prevalence among tribal population is about 10% for carriers and 0.5% for sickle cell disease patient.

On HPLC Sickle cell trait patient showed higher level of HbA_2 than the range of HbA_2 in normal person. Our findings do correlate with previous result. The higher level of HbA_2 is because of co-eluted glycated or otherwise modified HbS adduct[7, 8].

Sickle cell disease patient besides showing HbS values more than 70% showed raised HbF level. Raised HbF was also seen in central India and Orissa[9].

The diagnostic cut off level for HbA2 of 4.0% was set. Only 0.7% was diagnosed as Beta Thalassemia trait. This was due to surveillance carried in tribal area where the prevalence of sickle cell disorder is more.

Increase levels of HbA2 above 4% have been reported in absence of Beta Thalassemia, in a few significant cases of hyperthyroidism[10], in HIV infected patients treated with anti retroviral therapy [11, 12], and in the presence of megaloblastic anemia [13]. Thus clinical history, Red cell indices, peripheral smears do play a major role.

Three cases of Thalassemia major were identified they presented with marked increase of fetal hemoglobin> 85% with concomitant reduction in hemoglobin A.

Use of HPLC has helped in identification of compound heterozygous disorder. HbS-beta Thalassemia, HbE-beta Thalassemia. Though it was picked by conventional method, use of HPLC has helped in further sub characterization of these syndromes based on quantification of HbE, HbS and HbA levels [14].

Three patients had Hb band in SDG region and negative solubility test. On HPLC they were detected to have Hb-D Punjab. It is thus recommended that in all cases where HbS migration occurs in SDG region on electrophoresis HPLC should be definitely performed for further sub characterization of rare Hb variant [15].

Hb-D Punjab trait showed decrease A2 level (1.5-2.5%), the finding is consistent with

previous reports where similar ranges (0.9-2.5%) were reported[16]. It was postulated that the decrease in HbA_2 may be attribute to either co-elution with the HbA_0 or HbD Punjab peaks due to integration error or the mutation itself influencing the amount of delta chain[17].

The highest frequency of HbD Punjab is recorded in Punjab followed by Jammu and Kashmir[18]. Our case belonged to single family with their native place in Punjab.

HPLC avoids misidentification of two hemoglobin variants of HbD family HbD Punjab and HbD Iran. Both exhibit identical electrophoretic mobility's at alkaline ph. But on HPLC HbD Iran elute in A_2 and HbD Punjab in D windows. This situation is clinically important because HbD Punjab produce a significant sickling disorder when present in double heterozygous HbD-HbS form, where asHbD-Iran is clinically benign[19, 20].

Three patients of HbE heterozygous belonged to same Muslim family. HbE is commonly seen in Muslim family.

HPLC has $_2$some intrinsic problems. Some hemoglobin's co-elute in the HbA_2 window likeHbE, HbD Iran, and HbLepore.Differentiation on HPLC relies on the fact that in Thalassemia the highest HbA_2 level expected is 9%, in heterozygous states, HbD Iran is more than 40% while HbE is less than 40%. Alkaline electrophoresis shows migration of abnormal hemoglobin (HbD-Iran) in s/D/G position where as HbE migrates in the C/E/O position.

HPLC has been emerging as a method of choice in diagnosing hemoglobinopathies and Thalassemia. With automation and quantitative power, it appears to be a sensitive and accurate technique for direct identification and quantification of normal and abnormal hemoglobin fraction[21-26].

With requirement of only 5ul of blood sample, up to 100 samples can be simultaneously analyzed with each sample taking only 6.5 minutes of analysis.

Based on volume of workload, ease of handling, convenient data storage, local availability, expertise, less manpower use of HPLC in tertiary unit isideal. Retention time and percentage of variant hemoglobin can provide important clue in differentiating variant hemoglobin's eluting in the same window.

In Prevention and control program HPLC should be used as an additional method for identification of hemoglobin variant where the work load is more and automation is necessary. Our study had 9.11% Sickle cell trait patients. Early detection of trait will prevent occurrence of sickle cell disease in offspring. Detection of other variants is too important to avoid hematological abnormality. Findings must be supplemented by hemogram, family studies, hemoglobin electrophoresis.

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