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Pathology Section

Spectrum of Hemoglobinopathies and Thalassemias Diagnosed on HPLC in A Tertiary Teaching Hospital of Northern India

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ABSTRACT

Introduction: Hemoglobinopathies constitute an increasing global health burden. They are the most common genetic disorders in the World. As per WHO, the incidence is highest in the Middle East and Indian subcontinent. Various hemoglobin variants including HbF and HbA2 can be screened using a single, highly excellent method with the use of high performance liquid chromatography (HPLC).

Aim: The present study was carried out to diagnose hemoglobinopathies and thalassemias by the use of HPLC in a tertiary care teaching hospital of Northern India.

Materials and Methods: A prospective study of 22 months duration was conducted including 100 patients screened for the presence of thalassaemia or any other hemoglobin structural variants. All cases of microcytic hypochromic anemia (MCV <80 fl, MCH <27 pg) not responding to conventional treatment and with clinical suspicion of hemoglobinopathy were included in the present study. Patient witha history of recent blood transfusion of less than 3 months duration were excluded from the study.

Results: A total of 100 cases (57 males and 43 females) were included in the present study. The age group of patients ranged from 2 months to 40 years. Complete blood count, Red blood cell indices and peripheral blood examination were done in all the cases. Out of the 100 cases, 51 (51%) cases displayed abnormal hemoglobin fractions on HPLC of which. 42 (42%) cases were diagnosed as thalassemia trait, 4 cases (4%) as beta thalassemia major (HbF more than 75%), 2 cases as HbE (2%) and, 3 cases as HPFH (3%). 49 cases had a normal HPLC pattern.

Conclusion: HPLC is a rapid, accurate and useful method for diagnosing hemoglobinopathies. It serves as an important tool in diagnosing beta thalassemia traits especially in developing countries like India, where, the resources for detection of hemoglobinopathies are limited. Early diagnosis may help in proper and specific management.

Keywords: Chromatography, HbE, HbFH, Hemoglobin

INTRODUCTION

Liquid chromatography was first used as a method of separation of colored compounds in the 20th century. The term HPLC was coined by Csaba Horvath. Later, it was renamed as High Performance Liquid Chromatography (HPLC). HPLC has vast uses in clinical diagnosis of various diseases like hemoglobinopathies including thalassemias and disorders of body fluids which are related to glandular secretions [1,2].

Hemoglobinopathies are the most common genetic disorders reported in the World [3,4].The occurrence has been reported to be the highest in the Middle East and the Indian subcontinent. WHO claims to have an estimated 269 million carriers in the World. Globally, it is estimated that there are about 300,000 babies born every year with severe hemoglobin (Hb) disorders and 80% of these disorders do occur in the developing countries. In the Indian subcontinent the incidence of β-thalassemia major and hemoglobin E is high. Parts of South East Asia and the Mediterrian region have high prevalence of alpha thalassaemia [1,2,5-7]. As the awareness of these disorders is increasing more cases are being diagnosed early. Various Hb variants can be screened using a single, highly excellent method of HPLC. It is an automatic system with sample preparation, rapid analysis, better resolution and accurate identification of Hb variants [8,9].

AIM

The present study was conducted with the aim of diagnosing hemoglobinopathies and thalassaemias using HPLC method in a tertiary care teaching hospital of Northern India.

MATERIALS AND METHODS

This prospective study was carried out in the Department of Pathology, Teerthanker Mahaveer Medical College Hospital and Research Centre from January 2014 to October 2015. Institutional Ethical committee clearance was obtained for the study.

A total of 100 cases were screened for the presence of thalassemia or any other structural variants of hemoglobin. Informed consent was obtained in all the cases. All cases of microcytic hypochromic anemia (MCV <80 fl, MCH <27 pg), not responding to conventional treatment and with a clinically suspicion of hemoglobinopathy were included in the present study. Patients with a recent history of transfusion (less than three months prior to sample collection) were excluded from the study. A 5ml intravenous blood sample was collected in EDTA anticoagulant and then run on an automated hematology analyzer (Sysmex 800i) for complete blood counts (CBC) including RBC indices. Human hemoglobin was chromatographically separated by HPLC method into HbA2, HbF and other hemoglobin variants. Bio-Rad D10 instrument using the principle of ion-exchange high-performance liquid chromatography under the standard conditions specified by the manufacturer was used for estimation.

Most commonly occurring hemoglobin have established windows on the basis of characteristic hemoglobin retention time. After HPLC a chromatogram was obtained in all the cases which showed all the fractions of Hb, retention time, peak areas and the value of the fractions. These graphs were analyzed considering the concentration of Hb A2 and HbF. Variant II CDM software was used for the interpretation of the data thus obtained.

In small children where the blood sample was less than 500 L a manual pre-dilution using mixture of 1.0mL wash/diluent with 5 L of whole blood sample was done before processing for HPLC. Hb A2/F calibrators, normal and abnormal controls were run at the beginning of each sample tested.

CBC, red blood cell indices and peripheral blood examinations were carried out in all the cases. For diagnosing Beta thalassaemia trait the cut off value of Hb A2 of more than 3.9% was taken into consideration. The retention time for Hb A2 ranged between 3.30-3.90 minutes. Each chromatogram showed peaks of HbA0, A2, and HbF along with C window, D window, S window, and two minor peaks P2 and P3. Many Hb variants can be eluted in the same window. Considering the ethnicity of the patient, retention time and peak areas these Hb variants are diagnosed. The value of Hb A2 in beta –thalassaemia trait ranges between 3.5 to 9%. For diagnosing beta-thalassemia major, hemoglobin F (Hb F) values of 30% to 90% or more of the total hemoglobin were considered. For diagnosing delta/beta thalassaemia the HbF concentration considered was 5–15% of the total Hb value. For hemoglobin S (HbS) beta zero-thalassemia, higher concentrations of Hb F were recorded.

RESULTS

A total of 100 cases (57 males and 43 females) were included in the present study. The age group of the patients ranged from 2 months to 40 years. Out of these, 51 cases displayed abnormal hemoglobin fractions on HPLC. Of the total 51 cases of hemoglobin disorders, 32 were females and 19 males with a F:M ratio of 1.68:1. In females, the most common age group affected was 21-30 years, whereas in males, the most common age group affected was below 10 years. The pattern of Hb distribution observed is depicted in [Table/Fig-1].

In the present study, 42 (42%) cases had thalassaemia trait, 4(4%) beta thalassaemia major, 2 cases HbE (2%) and, 3 cases had HPFH (3%). 49(49%) cases had a normal HPLC pattern. Patients heterozygous for the Hb S gene showed hereditary persistence of fetal hemoglobin (HPFH). HPFH had a uniform intraerythrocytic distribution of HbF as compared to HbS/beta thalassaemia. In chronic anaemias, beta-thalassemia, and HPFH, the HbF values were greater than normal (2%). Peripheral blood smear showed microcytosis, hypochromia and presence of target cells in all the case. In most of the patients RBC count was raised. 35.71% patients of beta-thalassemia trait had Hb less than 9gm/dl and 28.57% patients had Hb ranging between 9-11 gm/dl. All patients had MCV less than 82 fl and MCH less than 27 pg. 83.33 % patients had MCHC more than 32% and 57.14% patients had RBC counts more than 4.8 million. Beta thalassemia major showed severe anemia, marked anisopoikilocytosis, microcytic hypochromic RBCs and polychromasia with presence of nucleated RBCs in the peripheral smear. Most patients had hemoglobin less than 7 gm/dl (35.71%). MCV was less than 82 fl in 100% patients

Hemoglobin pattern	Patients	
	n	Percentage (%)
Normal Hb Pattern	49	49
Beta Thalassemia Trait (BTT)	42	42
Thalassemia Major	4	4
HbE Homozygous	2	2
HPFH Heterozygous	3	3
Total	100	100

[Table/Fig-1]: Hemoglobin pattern among study subjects.

Hemoglobin Value	n (%)
<7 gm/dl	15 (35.71%)
7-9 gm/dl	12 (28.57%)
9-10gm/dl	08(19.04%)
>10gm/dl	07 (16.66%)
RBC Indices	
MCV	
<82 fl	42 (100)
82-92 fl	0 (0)
>92 fl	0 (0)
MCH	
<27pg	42 (100)
27-32 pg	0 (0)
>32 pg	0 (0)
MCHC	
<32 %	07 (16.66)
32-37%	35 (83.33)
RBC COUNT	
<3.8 million/cumm	04 (9.5)
3.8-4.8 million/cumm	14 (33.33)
>4.8 million/cumm	24 (57.14)

[Table/Fig-2]: Hemoglobin and RBC indices in patient with Beta thalassemia trait.

in our study group as this was our inclusion criteria itself. MCH was less than 27 in 100% cases. MCHC was mostly between 32-37% in 35 cases (83%) and less than 32 in 7 cases (16%). RBC count was more than 4.8 million/cumm in 57% of subjects. Cases of beta thalassemia major had HbF more than 75% [Table/Fig-2]. A comparison of the present study with that of different studies available in the literature is depicted in [Table/Fig-3].

DISCUSSION

Anaemia or low Hb concentration could be secondary to many factors including malnutrition, chronic blood loss or hereditary disorders such as hemoglobinopathies. Disorders of hemoglobin and thalassemia are autosomal recessive genetic disorders, mainly affecting the globin moiety of the Hb molecule. Alpha and thalassemia are the most common gene linked hemoglobin diseases in the World [3,10]. Thalassaemias and other hemoglobin variants were restricted to some particular geographical areas, caste, tribes and religion especially where marriages were confined to the same community and regions. However, now they are prevalent throughout the globe. The probable explanation to this is following increased migration of people from one place to other. Intercaste marriages are another reason for increase in the incidence and prevalence rates of hemoglobinopathies. The Indian population is composed of various castes and tribal groups, each with different genetic traits. There are numerous Hb variants in the Indian population many of which remain undetected due to lack of available infrastructure. Depending on the area of distribution, different hemoglobinopathies have been detected. β-Thalassaemia trait is the commonest Hb abnormality in the Indian subcontinent.In beta-thalassemia trait, the Hemoglobin A2 (Hb A2) values range between 3.5 to 9%. Low Hemoglobin, reduced MCV, MCH and raised RBC count suggest beta thalassaemia trait. For diagnosing beta–thalassaemia major the HbF values of equal to or more than 90% of the total Hb are considered. For diagnosing delta / beta thalassaemia trait (F–Thalassaemia) the concentration of HbF should be between 5-15% of the total Hb values. In hemoglobin S (Hb S)/beta zero-thalassemia, higher concentrations of Hb F do occur. In patients who are heterozygous for the Hb S gene have hereditary persistence of fetal hemoglobin (HPFH). HbE trait, HbE disease and HbE -β thalassaemia in India are more prevalent in the residents of West Bengal, Assam, Manipur and Nagaland [11-13].

In the present study a total of 100 cases (57 males and 43 females) were included. Of these 100 cases, 51 (51%) cases showed abnormal hemoglobin disorders. Among all the cases of abnormal hemoglobin, 32 were females and 19 were males. The most common hemoglobin disorder in our study was beta thalassemia trait (42%) which is in accordance with study of Chandrashekhar V but more when compared with Rao S and Hosseini S [1,14,15]. Beta thalassemia major was 4% in the present study which is in accordance with study done by Rao S and Chandrashekhar V. Hosseini S reported a higher incidence of beta thalassaemia major.

Age groups	Rao S et al., [1] (n-800)	Chandrashekhar V et al., [14] (n-543)	Hosseini S et al., [15] (n-1932)	Present study (n-100)
Beta Thalassaemia Trait	18.1%	37.9%	20.66%	42%
Beta Thalassaemia Major	2.9%	2.3%	12.87%	4%
HBE	2.5%	18.9%	4.6%	2%
HPFH	2%	1%	1.6%	3%

[Table/Fig-3]: Comparison with previous studies.

This discrepancy could due to the geographical distribution of hemoglobinopathies in the study population conducted. HPFH was observed to be 3% in the present study which is quite high when compared with those of the previous studies [9]. 2% cases of HbE was found in the present study which is in accordance with Rao S et al., and low when compared with Chandrashekhar V and Hosseini S [1,14,15]. Previous studies reported a rate of 20.66% of 20.66% for thalassaemia trait and 12.84% for β thalassaemia major. They also observed low MCV and MCH with normal iron level and normal Hb Electrophoretic pattern [15,16].

Authors in the previous studies have reported other Hemoglobin variants using HPLC method. Rao S Reported 247 of 800 (30.8%) cases to have abnormal hemoglobin variants including b- thalassaemia, HbS, HbE, HbD, Hb Q-India, Hb-Lepore, Sb- thalassaemia / HbFH, HbD-Iran, HbJ-Meerut and HbH disease [1]. The probable reason for this difference in the rates as well as the vast number of hemoglobin variants could be attributed to the fact that their study was conducted in a referral haematology centre receiving not only patients from all over India but also from abroad. Study by Khera et al., detected 110 cases of hemoglobinopathy including 87 (79.1%) of thalassaemia and 23 (20.9%) of other hemoglobinopathies including HbD, HbE, HbS and HbJ Oxford [17]. Study conducted in a Reference laboratory revealed that 12.6% cases (327 out of 1200 studied) had hemoglobinopathies. Majority of the patients had thalassaemia trait (8.9%). The other hemoglobinopathies detected in their study included - beta-thalassaemia major, thalassaemia intermedia, HbD Punjab, HbF, HbE, homozygous and heterozygous both, double heterozygous HbE-b thalassaemia trait, HbQ India, double heterozygous HbQ-India - b thalassaemia trait, HbS including HbS homozygous and HbS - b thal trait, HbJ Meerut, HbD – Iran and, HbLepore trait [2]. Gupta PK et al., in their two year study included 955 anaemic patients who were evaluated using HPLC for the diagnosis of hemoglobinopathies. A total of 137 patients (14.3%) showed different hemoglobin variants. Out of these 91(66.4%) were diagnosed to have beta – heterozygous thalassaemia, 5 (3.7%) as beta–homozygous thalassaemia (HbF 25–91%), 15(10.9%) as sickle cell trait, 2 (1.5%) as compound heterozygous state of sickle-β+thalassaemia and 3(2.2%)as having homozygous sickle cell anaemia [18].

Comparison with the previous studies show that Beta thalassaemia trait is the commonest disorder followed by Beta Thalassemia major, HBE and HPFH in this order [19,20]. Many Hb variants are prevalent in our population. This indicates that hemoglobinopathies are a common problem in the world today. The variation in the pattern of hemoglobinopathies can be attributed to the geographical area as well as increasing awareness among the patients and their relatives.

HbE trends to elute in the A2 window on HPLC and is the commonest hemoglobin variant in South East Asia and second most prevalent in the World. In HbE heterozygous individuals the HbE levels are below 40% and they are normal. HbE homozygous individuals have HbE levels more than 70% and are clinically characterized by anaemia, microcytosis, hypochromia and presence of target cells. It has been reported that of the cases of HbE disorders, HbE b- thalassaemia individuals had the lowest hemoglobin levels and the highest levels of HbF [2]. Double heterozygous Hb E – beta thalassaemia trait are important and symptoms resemble those of thalassaemia major. Also the reason of low HbE could be due to it being a thalassaemic hemoglobinopathy with unstable m-RNA. HbFH does not have substantial clinical significance [16]. It is a common practice among clinicians to give iron therapy in all anemic individuals leading halassemia syndrome or patients of other hemoglobin variants. Iron overload in such patients could be harmful in the long run [21]. In India, screenings before marriages are still considered a taboo. The best approach would be to target those patients attending the medical or Haematology OPD, the antenatal population and extended family members of known thalassemics/other hemoglobinopathies. Persons with a carrier state should be counselled regarding the disease nature (implications of being carrier which help in preventing birth of child with homozygous inheritance of hemoglobinopathies). The couple at risk should be counselled regarding the nature of the disease and the implications of being carriers. Options concerning birth control, including prenatal diagnosis and medical termination of pregnancy of the affected children should be informed. The couple should be informed of 25% recurrence risk and also advised to limit their family size. Analysis of global data reveals that effective screening methods in countries with high prevalence rates of thalassaemia significantly reduced the prevalence rates of hemoglobinopathies. Conducting a screening programme would be much cheaper to the exchequer rather than providing treatment to the affected.

The findings in the study present show HPLC as an excellent, powerful diagnostic tool for the direct identification of hemoglobin variants with a high degree of precision in the quantification of normal and abnormal hemoglobin fractions. Hb variants that are eluted in the same window can be differentiated according to the retention time and the percentage of different hemoglobins obtained.

CONCLUSION

Nutritional deficiency is a major cause of anemia. Abnormal hemoglobin as a cause of anemia should also be considered, since morbidity and mortality is higher in homozygous conditions of hemoglobinopathies. Detailed investigation for cause of anemia and, premarital and antenatal screening are helpful in detecting abnormal hemoglobin disorders and identifying more carrier states of different hemoglobinopathies. Combined approach of primary and secondary prevention needs to be followed. It would prove to be cost-effective if the birth of a child with genetic homozygous inheritance disease is prevented. Furthermore, there is an added advantage of detection and proper management of hemoglobinopathies and its variants.

HPLC has the advantage for screening and detection of various hemoglobinopathies over other tests by providing rapid and accurate results. HPLC can detect and measure HbF and HbA2 in a single system. It provides a superior resolution, is automated and internal sample preparation is possible. It is important especially when the incidence of beta thalassemia traits is higher in developing counties like India, where there are limited resources available for early diagnosis.

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REFERENCES

[1] Rao S, Kar R, Gupta S K, Chopra A, Saxena R. Spectrum of hemoglobinopathies diagnosed by cation exchange HPLC & modulating effects of nutritional deficiency anaemias from north India. 2012; 132: 513-19.

[2] Sachdev R, Dam AR, Tyagi G. Detectionof Hb variants and hemoglobinopathies in Indian population using HPLC: Report of 2600 cases. *Ind J Pathol Microbiol*. 2010;53:57-62.

[3] Modell B, Darlison M. Global epidemiology of hemoglobin disorders and derived service indicators. *Bull World Health Orga*n. 2008;86:480-87.

[4] Weatherall D. The inherited disorders of hemoglobin: an increasingly neglected global health burden. *Indian J Med Res*. 2011;134(4):493–97.

[5] Madan N, Sharma S, Sood SK, Colah R, Bhatia HM. Frequency of β-thalassemia trait and other hemoglobinopathies in northern and western India. *Indian J Hum Genet*. 2010;16:16-25.

[6] Patel AP, Naik MR, Shah NM, Sharma NP, Parmar PH. Prevalence of common hemoglobinopathies in Gujarat: An analysis of a large population screening programme. *Natl J Coom Med.* 2012;3(1): 112-16.

[7] Kishore B, Khare P, Gupta RJ, Bisht S, Majumdar K. Hemoglobin E disease in north Indian population: A report of 11 cases. *Hematology.* 2007;12:343-47.

[8] Bhalodia JN, Oza HV, Modi PJ, Shah AM, Patel KA, Patel HB. Study of hemoglobinopathies in patients of anemia using high performance liquid chromatography (HPLC) in Western India. *Natl J Community Med*.2015; 6(1):35-40.

[9] Patel U, Shrivastav A, Joshi J R, Agnihotri A S, Kaur A, Thakkar B. Detection of hemoglobinopathies and thalassemias in population of Gujarat state using HPLC: Analysis of 2022 cases. *Natl J Community Med.* 2012; 4(2): 80-84.

[10] Balgir RS. Genetic epidemiology of the three predominant abnormal hemoglobins in India. *J Assoc Physicians India* 1996;44:25-28.

[11] Joshi H, Subbarao SK. Prevalence of G-6-PD deficiency and sickle-cell hemoglobin carriers in malaria endemic tribal dominated districts-Mandla and Jabalpur, Madhya Pradesh. *Indian J Malariol*. 2001;38:99-104.

[12] Balgir RS. Aberrant heterosis in hemoglobinopathies with special reference to beta-thalassemia and structurally abnormal hemoglobins E and S in Orissa, India. *J Clin Diagn Res*. 2007;1:122-30.

[13] Balgir R S. Spectrum of hemoglobinopathies in the state of Orissa, India: A ten years cohort study. *J Assoc Physicians India*. 2005;53:1021-26.

[14] Chandrashekar V, Soni M. Hemoglobin disorders in south India. *ISRN Hematol*. 2011;2011:748939. Doi:10.5402/2011/7489-39.

[15] Hosseini S, Kalantar E, Dorgalaleh A. A long term screening of Iranian populations with thalassaemia and hemoglobinopathies, *Br Biomed Bull*. 2014; 2(4):669-76.

[16] Philip J, Sarkar RS, Kushwaha N. Microcytic hypochromic anemia: Should high performance liquid chromatography be used routinely for screening anemic and antenatal patients?*Indian J Pathol Microbiol*. 2013;56:109-13.

[17] Khera R, Singh T, Khurana N, Gupta N, Dubey AP. HPLC in characterization of hemoglobin profile in thalassemia syndromes and hemoglobinopathies: clinicohematological correlation. *Indian J Hematol Blood Transfus*. 2015; 31(1):110-15.

[18] Gupta PK, Kumar H, Kumar S, Jaiprakash M. Cationexchange high performance liquid chromatography for diagnosis of hemoglobinopathies. *MJAFI*. 2009; 65(1): 33-37.

[19] Gupta V, Shukla J, Tilak V, Bhatia B. Spectrum of hemoglobinopathies in eastern Uttar Pradesh. *Indian J Pediatr*. 2009;76:857.

[20] Baruah MK, Saikia M, Baruah A. pattern of hemoglobinopathies and thalassemias in upper Assam region of North Eastern India: High performance liquid chromatography studies in 9000 patients. *Ind J of Pathol and Microbiol*. 2014; 57(2): 236- 43.

[21] Tyagi S, Saxena R, Choudhry VP. HPLC--how necessary is it for hemoglobinopathy diagnosis in India? *Ind J Pathol Microbiol*. 2003;46:390-93.

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