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Original Research Article

A Study of Different types of Hemoglobinopathies in Pediatric Population with Anemia by High Performance Liquid Chromatography in a Tertiary Care Centre

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Abstract

Background: Hemoglobinopathies are the most common inherited red cell disorders worldwide. Identification of these disorders is immensely important epidemiologically and for improved management protocols. Among these Hemoglobinopathies, Thalassemia and Sickle Cell Anemia constitute major public health problems.

Aim: To study the prevalence of Hemoglobinopathies in anemic pediatric population by using HPLC.

Materials and Methods: This study was done at our tertiary care center, from January 2021 to July 2022. Hematological indices were derived from hematology analyzer (Mindray BC-5150) and Hemoglobin Electrophoresis was carried out by D-10 (BIO-RAD).

Results: Out of 378 cases 33 (8.73%) cases had Hemoglobinopathies. The most predominant hemoglobinopathy was of Thalassemia cases 27 (7.76%) followed by Sickle Cell disorders cases 4(1.06%) with slight male preponderance, 20 were male and 13 were female.

Conclusion: An extensive screening of the population is important to assess the prevalence of hemoglobinopathies, which will help in identification of carriers and take adequate therapeutic and preventive measures. Cation Exchange HPLC is emerging as one of the best methods for screening and detection of various Hemoglobinopathies with rapid, reproducible and precise results.

Keywords: Hemoglobinopathies, HPLC, Anaemia.

Introduction

Since 1956 to 1961 the chemical structure of hemoglobin was identified and complete sequencing of Globin chain was described [1]. After that continuous efforts by different science scholars and researchers, till date more than 900 hemoglobin (Hb) variants are identified and worldwide, an estimated 150 million people carry different types of hemoglobin variants [2]. Within the primary structure of hemoglobin, four polypeptide chains are attached to a haem molecule and every chain is arranged in a series of eight helical segments joined by short non-helical segments, is referred as secondary structure. In hemoglobin A, two α chains combine with two β- chain ($\alpha_2 \beta_2$) while in hemoglobin A2 (α2 δ 2) and hemoglobin F (α2 γ 2) β- chains are replaced by δ chains and γ chains respectively [3].

Most common human single gene disorder is different types of mutations in hemoglobin chains. About 5.2% of the world population (and more than 7% of pregnant females) carry a mutated hemoglobin variant. Globally, about 1.1% of couples are at risk for having children with Hemoglobinopathies of which 2.7 per 1000 conceptions are actually having disease. Worldwide contribution of Hemoglobinopathies in mortality of children aged less than five years is around 3.4% [4].

Among these Hemoglobinopathies, Thalassemia and Sickle Cell Anaemia constitute major public health problems. The inheritance of Thalassemia is an autosomal recessive and it is characterized by the absence or reduction of one or more of the globin chains of hemoglobin. The different types of variants are result from substitution of one or more amino acids in the globin chains of the hemoglobin molecule [5]. In different regions of India the frequency of Beta-Thalassemia Trait (βTT) in India is varied< 1 to 17%, with an average of 3.3% [6].

Like Thalassemia, Sickle Cell Anaemia is also an autosomal recessive disorder. In India the prevalence of Sickle Cell Anaemia is around 4.3% which varies in different regions.[7] Due to mutation in β-globin protein encoding gene, Red Blood Cells (RBC) to become sickle shaped with reduced oxygen carrying capacity [7].

Patients having Hemoglobinopathies are presented with wide range of clinical manifestation which may vary from asymptomatic states to severe, lifelong, transfusion-dependent anemia with multi organ involvement and severely reduced life expectancy. So, early diagnosis of these disease are exceeding important towards the patient for acquiring proper treatment modalities and minimization of complications. At community level measurement of burden of Hemoglobinopathies are also important. The aim of this study was to determine the prevalence of Hemoglobinopathies in anemic pediatric patients by using HPLC.

Material and Method

This longitudinal study was carried out at Rajshree Medical Research Institute and tertiary care hospital of western Uttar Pradesh, after getting approval from Institutional Ethics Committee. The data was collected from January 2021 to June 2022 of those patients who had been attended OPD and admitted in IPD of pediatric department with the symptom of anemia. Non cooperative patients, patients lost in follow up and poorly sampled blood were excluded from study.

EDTA treated venous blood samples were received in hematology laboratory and analyzed by hematology analyzer (Mindray BC-5150) for Complete Cell Count and red blood cell indices. After that, blood samples of anemic patients were further processed for HPLC (High Performance Liquid chromatography) by using D-10 (BIO-RAD) instrument. The demographic details of patients and laboratory findings are collected for study purpose.

Collected data was transcribed into MS Excel datasheet and analysed by using SPSS (version 19) software. Study variables depending on the data type were summarized using appropriate measure of central tendency (mean) and dispersion - standard deviation (SD). Categorical variables were expressed as frequencies and percentages. Pearson's Chi-square test was done for comparison of categorical variables. Value of $p \leq 0.05$ was considered statistically significant along with the 95% confidence interval for the test statistic was computed.

Result

In present longitudinal study a total of 378 anemic patients of pediatric age group were included, of them 233 (61.64%) males and 145 (38.36%) females. The mean age (±SD) of study population was 5 year 6months (±2 year 10 months) and mean age (±SD) of male and female population were 5 year 3 months (±2 year 11 months) and 5 year 9 months(± 2year 7 months) respectively.(Table-1)

Table 1: Gender distribution of total cases

Gender	No. of Cases	Mean±SD
Male	233 (61.64%)	5 year 3 months ±2 year 11 months
Female	145 (38.36%)	5 year 9 months± 2year 7 months
Total	378	5 year 6months ±2 year 10 months

The etiology of anemia of these patients was identified and maximum numbers of 144 (38.09%) cases were presented with iron deficiency anemia. Other major causes like Macrocytic Anemia, Dimorphic Anemia, Hemoglobinopathies, Anemia due to infections and Leukemia were also identified. A total 33 (8.73%) cases of Hemoglobinopathies, 27 (7.76%) cases of Thalassemic disorders and 4(1.06%) cases of Sickle Cell Anaemia were identified, of them 20 were male and 13 were female. (Table-2)

Table 2: Different Etiologies of anemia and their distribution in study population

Etiology		Male	Female	Total	
Iron Deficiency Anemia		88	56	144	
Macrocytic Anemia		65	34	99	
Dimorphic Anemia		44	27	71	
Hemoglobinopathies	Thalassemia Major	8	5	13	33
	Thalassemia Trait	9	7	16	
	Sickle Cell Anaemia	3	1	4	
Anemia due infection		15	12	27	
Leukemia/Aplastic Anemia		1	3	4	
Total		233	145	378	

Out of total 13 cases of β Thalassemia Major 9 cases were seen in age group of <1 year of age and remaining 4 cases were diagnosed in age group of 1-2 years . 6 cases of β Thalassemia Trait were diagnosed in age group of 1-2 years followed by 4 cases in age group 5-6 year, 3 cases in age group 3-4 years and 3 cases in age group 7-8 years. All 4 cases of Sickle Cell Disease were diagnosed in four different age groups like <1year, 1-2 year, 7-8 year and 9-10 year.(Table-3)

Table 3:Showing Showing Distribution of different Hemoglobinopathies according to the age of the patient

HPLC interpretation	AGE							Total cases
	<1 year	1-2 year and 11 months	3-4 year and 11 months	5-6 year and 11 months	7-8 year and 11 months	9-10 year and 11 months	>10 years	
β Thalassemia Major	9	4	0	0	0	0	0	13
β Thalassemia Trait	0	6	3	4	3	0	0	16
Sickle cell disease	1	1	0	0	1	1	0	4
Total	10	11	3	4	4	1	0	33

In this study the mean hemoglobin concentration was 9.21±1.58 gm/dl. Hemoglobin concentrations in different type of anemia were different. In Thalassemia Major patients mean hemoglobin concentration are 5.58±0.97 gm/dl while hemoglobin in Thalassemia Trait, Sickle Cell Anemia and Anemia without Hemoglobinopathies are 9.22±0.79, 9.8±0.7 and 9.34±1.47 respectively. Hemoglobin in Thalassemia Major is much lower than other causes of Anemia ($p<0.0001$).(Table 4)

Table 4: Anaemia patients showing the different fraction of hemoglobin variants

HPLC Interpretation	**Hemoglobin%**	**HbA%**	**HbA_2%**	**HbF%**	**HbS%**
β Thalassemia Major	5.58 ±0.97%	20.12 ±13.5%	3.13 ±0.84%	71.27 ±13.07%	-
β Thalassemia Trait	9.22 ±0.79%	86.24 ±1.88%	5.17 ±0.41%	1.93 ±0.4%	-
Sickle Cell Disease	9.8 ±0.7%	5.97 ±1.45%	3.85 ±0.72%	13.95 ±2.18%	71.02 ±3.64%
Anemia without Hemoglobinopathies	9.34 ±1.47%	84 ±0.76%	2.53 ±0.18%	0.75 ±0.07%	-

HbA concentration in Thalassemia Major, Thalassemia Trait, Sickle Cell Anemia and other conditions are 20.12±13.5%, 86.24± 1.88%, 5.97 ±1.45% and 84±0.76% respectively. Comparing the fraction of HbA in Thalassemia Major with other causes of anemia, the value of HbA is significantly reduced in Thalassemia Major Cases (p value<0.0001). HbA2 concentration in Thalassemia Major, Thalassemia Trait, Sickle Cell Anemia and other causes of anaemia are 3.13±0.84, 5.17±0.41, 3.85±0.72 and 2.53±0.18 respectively. HbA2 concentration in Thalassemia Major is significantly increased as compared with other causes of anemia (p value<0.0001). The fraction of HbF in Thalassemia Major, Thalassemia Trait, Sickle Cell Anemia and other conditions are 71.27±13.07, 1.93±0.4, 13.95 ±2.18% and 0.75±0.07 respectively. Comparing the fraction of HbF in Thalassemia Major with other causes of anemia, the value of HbF is significantly increased in Thalassemia Major Cases (p value<0.0001). (Table 5)

Table 5: Comparison between different types of hemoglobin variants in Thalassemia Major patients with anaemia without hemoglobinopathies

	Thalassemia Major	**Anemia without Hemoglobionpathies**	**p Value**
Hemoglobin %	5.58±0.97	9.34±1.47	p<0.0001
HbA	20.12±13.5	84±0.76	p<0.0001
HbA2	3.13±0.84	2.53±0.18	p<0.0001
HbF	71.27±13.07	0.75±0.07	p<0.0001

The concentration of HbS in Sickle Cell Anemia is 71.02±3.64, which is significantly increased as compared to with other causes of Anemia.($p<0.0001$)

Table 6: Comparison between different types of hemoglobin variants in Sickle cell anaemia patients with anaemia without hemoglobinopathies

	Sickle Cell Anemia	Anemia without Hemoglobionpathies	p Value
Hemoglobin %	9.8±0.7	9.34±1.47	p=0.532
HbA	5.97 ±1.45%	84±0.76	p<0.0001
HbA2	3.85±0.72	2.53±0.18	p<0.0001
HbF	13.95±2.18	0.75±0.07	p<0.0001
HbS	71.02±3.64	-	p<0.0001

Discussion

Anemia in pediatric age group is a major public health concern in both developed and developing countries. According to World Health Organization (WHO) the prevalence of anemia among children of 6–59 months was 42.6% globally in 2011, of which, 53.8% seen in South East Asia and 59% in India [8] Reports of Fourth National Family Health Survey (NFHS) (2016), clearly mentioned the burden of anemia is 58.6% among 6 to 59 months of children in India [9].

Hemoglobinopathies are one of the considerable causes of anemia in pediatric age group and these disorders are characterized by the presence of structurally defective hemoglobin (Hb) due to abnormalities in the formation of globin moiety of the molecule [10]. There are two major groups of Hemoglobinopathies: Thalassemia syndromes and structural hemoglobin variants (abnormal hemoglobin) [11]. According to World Health Organization (WHO) a total 5% of the world population is a carrier of Hemoglobin disorders [12] In India the frequency of distribution of defective gene is around 4.2% and over 12,000 infants born each year with a clinically significant hemoglobinopathy [13]. The frequency of Beta-Thalassemia Trait (βTT) in India has been reported to vary from less than 1 to 17% depending on the region studied, with an average of 3.3% [6]. The prevalence of Sickle Cell Disease is 4.3% while the occurrence of Hemoglobin E patients in India is common in the north-eastern region with a prevalence of 10.9% [14]

In our study out of 378 cases only 33(8.73%) cases were detected with abnormal hemoglobin variants. Of them 20(60.60%) were males and remaining 13(39.40%) females. These findings are comparable to studies conducted by Bush A *et al* [15] and Shankar R *et al* [16].

β Thalassemia Trait is the most prevalent Hemoglobinopathy in our country [17-21]. Similar to that in our study 16 cases (48.48%) of β Thalassemia Trait were identified and this was parallel to the findings to other studies conducted by Bush A *et al* [15] and Khera *et al* [22]. The HbA_2 level in Beta Thalassemia Trait is elevated which range from 4% to 8% and in our study the levels of HbA_2 was constantly elevated with a mean of 5.17%±0.41%. This finding was similar to the study by Khan H *et al* [23] with HbA_2 level of 5.57±0.63 for Beta Thalassemia Trait. Different authors have established different cutoff values for HbA2 for diagnosis of Beta Thalassemia Trait, which ranges from 3.5% to 4%. It has been recommended that each laboratory should establish individual normal ranges [24] In our study the cut off value considered for Beta Thalassemia Trait was >4.0%.

In the present study, majority of Beta Thalassemia Major Cases were seen in the age group of 0-1 years followed by 1-2 year and 11 months. This was comparable to the study of Khan H *et al* [23] in which all the cases of Beta Thalassemia Major were seen with an average age of presentation being 1.6 years. The value of HbF in Beta Thalassemia Major groups were 71.27±13.07 which was also comparable in study of Barbaria SS *et al* [25] with HbF value of 76.3 . However, this study showed relatively lower values when compared to the study conducted by Baruah MK *et al* [26] and Khan H *et al* [23] with the HbF values of 88.6±6.2, 92.35±4.83 respectively.

In India Sickle Cell Disease is mainly prevalent in tribals and certain ethnic groups in central part of country. Family history, careful PBS examination, Sickling Test and Hb variant analysis by HPLC are useful for the diagnosis of Sickle Cell Disease [27] In the present study, 4 cases were detected with Sickle Cell Disease and all the 4 cases were sickle cell homozygous. Sickle cell homozygous had a peak in S window with HbS level of 71.02±3.64, increased HbF value of 13.95±2.18 and HbA_2 levels ranging from 3.85±0.72.

These findings are comparable with Vasaikar M *et al* [28] who reported HbS values >70% and increased HbF value (5-20%). In our study 2 patients are seen in the age group of 0-2 years and 2 patients are seen in the age group of 7-10 years of age with mean hemoglobin level of 9.8±0.7. In sickle cell disease patients, there were significantly higher levels of HbA2, HbF and HbS and significantly lower levels of HbA. This is consistent with various studies done by Eman A *et al* and Cotton F *et al* [29,30].

Proper screening and early diagnosis of Hemoglobinopathies is vital to their management and treatment. Improper screening or delay results in higher mortality rates among these patients. Several screening techniques were developed in the early 1970s but unfortunately, many such methodologies proved to be unreliable since they gave false positive or negative results that led to wrong treatment and confusion. In order to standardize laboratory techniques and interpretation for SCD screening, a Hemoglobinopathy Reference Laboratory (HRL) was created at the Centers of Disease Control (CDC) [31].

Cation Exchange HPLC is emerging as one of the best methods for screening and detection of various Hemoglobinopathies with rapid, reproducible and precise results [32]. It has the advantage of quantifying Hb F and Hb A2 along with hemoglobin variant screening in single and highly reproducible system. The simplicity of the automated system with internal sample preparation, superior resolution, rapid assay time, and accurate quantification of hemoglobin fractions makes this an ideal methodology for routine clinical laboratory [33]. The major limitation of HPLC is high cost and requirement of knowledge, skill, and experience for interpretation of results. Alpha Thalassemia, normal HbA2 Beta Thalassemia, and other rare Hemoglobinopathies that elute with similar retention values on HPLC cannot be ruled out. Hence, the reports must carry a note to interpret the findings with reference to hemogram, family/sibling studies, other confirmatory techniques, and molecular studies when necessary.

Conclusion

In our country major cause of anemia is nutritional deficiencies which can be treated by medications. Abnormal hemoglobin as a cause of anemia should also be considered as morbidity and mortality is higher in homozygous conditions of Hemoglobinopathies. Continuous awareness programs, mass screening of the population especially childbearing age and school going children will help in early detection of heterozygous states. This can in turn with

proper genetic counseling help in reducing the morbidity and mortality.

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