ORIGINAL RESEARCH

Detection of abnormal haemoglobin variants and its characterization among anaemicsby high performance liquid chromatography (HPLC): A prospective study from North India

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ABSTRACT

Background: Hemoglobinopathies are one of the most common inherited blood illnesses in India and are one of the country's most serious public health issues. In India, it is estimated that there are approximately 45 million carriers and 15,000 babies born with hemoglobinopathies per year. The carrier rate of hemoglobinopathy in various Indian demographic groups ranges from 3 to 17 percent. Automated cation-exchange High Performance Liquid Chromatography (HPLC) has surfaced as an outstanding screening tool for identifying this abnormal hemoglobin or thalassemic conditions. So, the purpose of this study was to identify common haemoglobin disorders, in anaemia patients using HPLC.

Methods: The present hospital based prospective study was carried in the Department of Pathology, over a period of 12 months from September 2019 to October 2020. The present study included subjects (40 years or below) attending OPD/admitted to IPD having anaemia. Consecutive sampling method was used to enrol the study subjects, so a total of 945 patients were enrolled.4 mL of venous blood was obtained from subjects. On an automated haematology analyser red cell indiceswere assessed. The HPLC method for chromatographic separation of human haemoglobin was used to investigate haemoglobin variations. All tests were performed at a 5% level of significance.

Results: Out of 945 subjects, the analysis was carried out in 618 subjects with hemoglobinopathies and it was observed that 36.3% of subjects had Sickle cell trait (343/945), 13.1% had Sickle cell disease (124/945), 11.0% had Thalassemia trait (104/945), 4.7% had S-Beta Double heterozygous (45/945).In all age groups the Sickle cell trait was commonest hemoglobinopathy. The hemoglobinopathies Hb D (1/104) and Hb E (1/104) were observed only in the age group of 31-40 years. In the present study, there were 63.3% of subjects with hemoglobinopathies were female (391/618) and 27.7% were males (227/618).In both sexes the Sickle cell trait was commonest hemoglobinopathy.The hemoglobinopathies Hb D (1/391) and Hb E (1/391) were observed only in females. In the present study the highest mean Haemoglobin was observed in the Thalassemia trait (10.2 ± 2.3 g/dl) and lowest mean haemoglobin was noticed in Thalassemia major (4.8 ± 3.5 g/dl).Similarly, the highest RBC count was

observed in the Thalassemia trait (4.7 ± 0.9 x 106 mm3) and lowest RBC count was noticed in Thalassemia major (2.5 ± 1.4 x 106 mm3).In the present study RDW-CV (%) was raised among subjects with hemoglobinopathies. The haemoglobin variant Hb A (%) was highest as 89.1 ± 2.7% in Thalassemia trait and lowest as 5.2 ± 10.5% in Sickle cell disease.

Conclusion: Nutritional deficiencies, which can be remedied with drugs, are the leading cause of anaemia in India. Anemia caused by abnormal haemoglobin should also be evaluated, as morbidity and mortality are significant in homozygous haemoglobinopathies.

Keywords: High performance liquid chromatography, Hemoglobin, Sickle cell anemia, Nutritional deficiencies, Thalassemia

INTRODUCION

Hemoglobinopathies are one of the most common inherited blood illnesses in India and are one of the country's most serious public health issues. In India, it is estimated that there are approximately 45 million carriers and 15,000 babies born with hemoglobinopathies per year. The carrier rate of hemoglobinopathy in various Indian demographic groups ranges from 3 to 17 percent. As a result, in India, the prevalence of hemoglobinopathies is enormous. In India, the frequency of sickle cell trait ranges from 0 to 18 percent in the north-east part of the country, 0 to 33.5 percent in the western region, 22.5 to 44 percent in the central region, and 1 to 40 percent in the southern parts. Hbs gene prevalence is estimated from 0.031 to 0.41 percent, with considerable variation in Hbs trait prevalence seen in demographic groups across limited geographical regions [1,2].

The World Health Organization (WHO) considers thalassemia to be the most common genetic blood disorder in the world, affecting over 60 countries. 3 to 10 percent of the world's population is carriers of the thalassemia gene, and nearly 250 million people are heterozygous for beta thalassemia, and at least 2,000,000 affected homozygotes are born each year, according to some reports. Delhi has the greatest prevalence of beta thalassemia, followed by Sindh, Punjab, Tamil Nadu, South India, and Maharashtra [3]. Automated cation-exchange High Performance Liquid Chromatography (HPLC) has surfaced as an outstanding screening tool for identifying this abnormal hemoglobin or thalassemic conditions [4]. Given growing international understanding and mass screening programmes implemented at all levels by the health sector, laboratory personnel's responsibilities in detecting and preventing this disease have greatly increased. An automated system is an effective approach for a routine diagnostic facility because it reduces the amount of labour and expert attention required for internal sample preparation, resulting in higher quality, faster assay performance, and more precise characterization. The purpose of this study was to identify common haemoglobin disorders, assess their prevalence, and investigate abnormal haemoglobin variants in anaemia patients using a High-Performance Liquid Chromatography (HPLC) BIO-RAD D-10 Dual Program Analyzer in Delhi, in order to aid in prevention, early diagnosis, and better management policies and procedures.

MATERIALS and METHODS

STUDY SETTING AND DESIGN

The present hospitalbased prospective study was carried out in the Department of Pathology, Government Institute of Medical Sciences, Greater Noida, Uttar Pradesh, India over a period of 12 months from September 2019 to October 2020, after obtaining ethical approval from Institutional Ethics Committee (IEC/IRB No.AIHFEC/04/023; Ahalia International Foundation Ethics Committee, Kozhippara).

STUDY SUBJECTS AND SAMPLE SIZE

The present study included subjects (40 years or below) attending OPD/admitted to IPD having anaemia (haemoglobin < 11gm%), hepatosplenomegaly, detection of sickle-shaped cells on peripheral smear, positive sickling solubility test, positive fetal fraction test, history of more than five blood transfusions in the absence of trauma or any clinical morbidity, not responding to conventional treatment, clinically suspected cases of hemoglobinopathy, antenatal, and other cases coming for thalassemia screening. Prior to enrolling subjects into the study, written informed consent was obtained either from patients or relatives after explaining in detail the purpose of the study, and a consecutive sampling method was used to enrol the study subjects, so a total of 945 patients were enrolled in the study during the defined study duration. There were no exclusion criteria.

DATA AND CULTURE SAMPLE COLLECTION

During OPD hours or after admission, 4 mL of venous blood was obtained from subjects using all aseptic precautions. 2 mL of blood was collected in a 4% K2 EDTA (Ethylene diamine tetra-acetic acid) anticoagulant bulb, and serum was isolated for further processing from the remaining 2 mL. On an automated haematology analyser (Sysmex KX 21), red cell indices [Hb (g/dL), RBC x 10^6cumm, MCV (fL), MCH (pg), MCHC (g/dL), and RDW] were assessed. The HPLC method for chromatographic separation of human haemoglobin [5,6,7] was used to investigate HbA2, HbF, and other haemoglobin variations. The Variant Hemoglobin Testing System (Variant II Beta Thalassemia Short Program, Bio-Rad Laboratories Inc., Hercules, CA, USA) was utilised in this work under the manufacturer's experimental protocols [8]. β -thalassemia short programme cation-exchange HPLC, which operates by adsorbing positively charged haemoglobin to a negatively charged stationary phase and eluting them with a mobile phase at a rate proportional to their affinity for the stationary phase. The samples (2 ml) are introduced into the analysis stream and separated by the cation exchange cartridge using a phosphate ion gradient created by mixing two buffers of varying ionic strengths to elute the various haemoglobins. The eluent from the cartridge is monitored as it travels through the photometer cell using a dual wavelength filter photometer. At 415 nm, changes in optical density are recorded. The effects of combining buffers of different ionic strengths are corrected by a secondary filter at 690 nm. The data is processed, and a report is generated with a chromatogram showing the individual peaks in defined windows, along with pertinent details like retention time, relative percentage, and area. By comparing the migration patterns of the test samples to those of known adult and foetal controls, the migration patterns of the test samples were deciphered.

STATISTICAL ANALYSIS

The data was entered into an MS Excel spreadsheet and the analysis was done using the Statistical Package for Social Sciences (SPSS) version 28. Categorical variables were presented in number and percentage (%) and continuous variables were presented as mean ± SD. Normality of data were tested by Kolmogorov-Smirnov test. If normality was rejected, then a non-parametric test was used. The chi-square test and the T test were used to find the difference between dependent and independent variables. All tests were performed at a 5% level of significance; thus, an association was significant if the p value was less than 0.05.

RESULTS

In present study, 945 subjects were screened for hemoglobinopathies and it was observed that 36.3% of subjects had Sickle cell trait (343/945), 13.1% had Sickle cell disease (124/945), 11.0% had Thalassemia trait (104/945), 4.7% had S-Beta Double heterozygous (45/945).Thalassemia major (2/945), Hb D (1/945) and Hb E (1/945) were observed among

0.2%, 0.1% and 0.1% of subjects respectively. The 34.6% of subjects (317/945) were having no hemoglobinopathies (Figure 1).

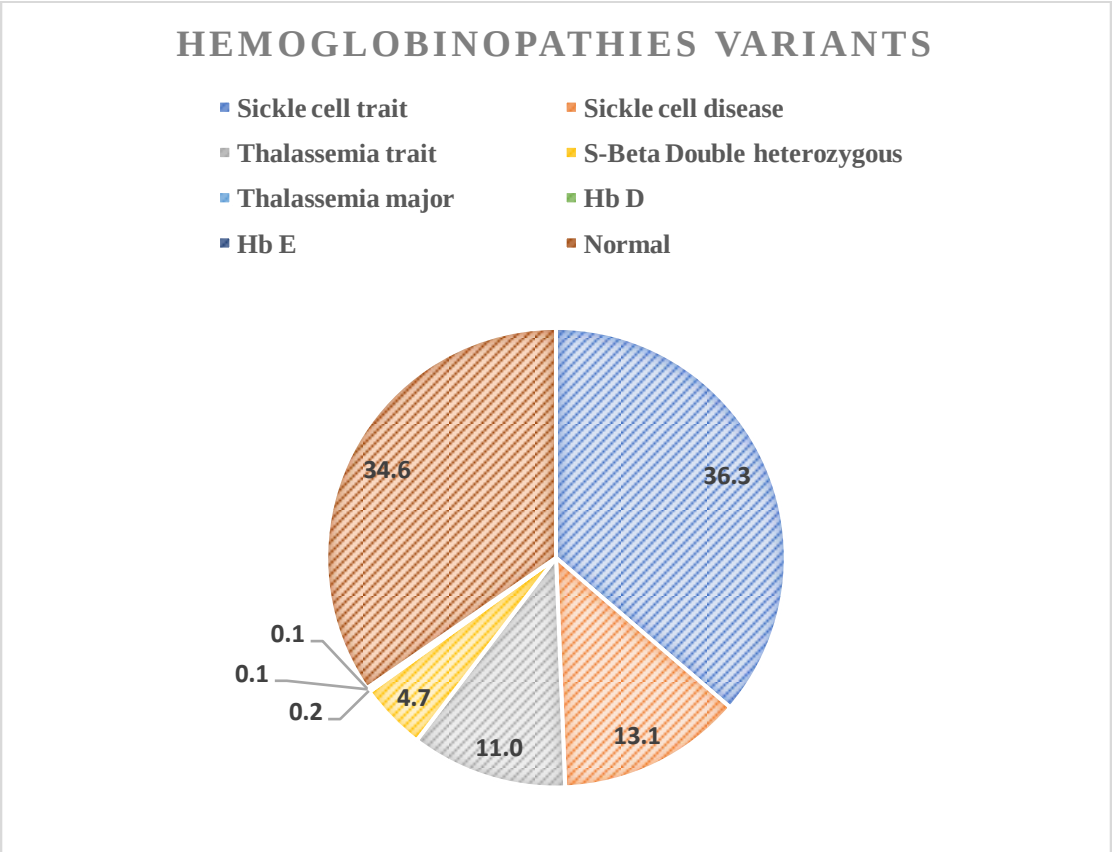

Figure1. Distribution of hemoglobinopathies variants detected by HPLC among screened subjects (N=945).

Out of 945 subjects, the analysis was carried out in 618 subjects with hemoglobinopathies.In present study the subjects belonging to age group of 0-10 years were 10.4% (65/618),44.2% (273/618) in the age group of 11-20 years, 28.6% (177/618) in the age group of 21-30years and 16.7% (104/618) of subjects were in the age group of 31-40 years. In the age group 0-10 years, Sickle cell trait (25/65) was most common hemoglobinopathy followed by Sickle cell disease (38/65) and Thalassemia major (2/65). In the age group 11-20 years,Sickle cell trait (134/273) was commonest hemoglobinopathyfollowed by Sickle cell disease (81/273), Thalassemia trait (38/273) and Double heterozygous for thalassemia and sickle cell (21/273). In the age group 21-30 years, again the most common hemoglobinopathy was Sickle cell trait (108/177), followed by Sickle cell disease(4/177), Thalassemiatrait (48/177) and Double heterozygous for thalassemia and sickle cell (18/177). In the age group 31-40 years, themost common hemoglobinopathy was Sickle cell trait (77/104), followed by Sickle cell disease (1/104), Thalassemia trait (18/104) and Double heterozygous for thalassemia and sickle cell (18/104). The hemoglobinopathies Hb D (1/104) and Hb E (1/104) were observed only in the age group of 31-40 years (Figure 2).

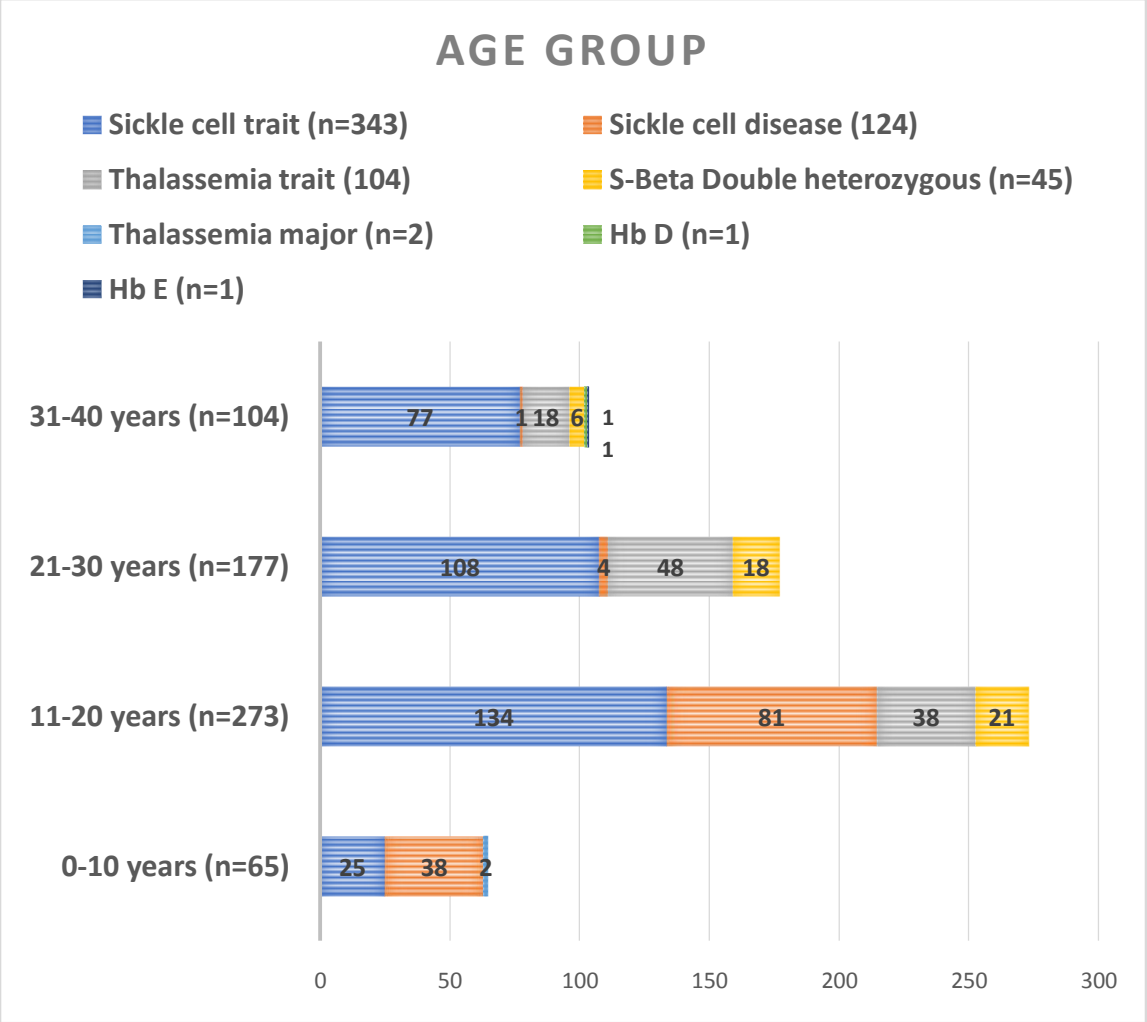

Figure2. Age wise distribution of hemoglobinopathies variants detected by HPLC among confirmed subjects (N=618).

In the present study, there were 63.3% of subjects with hemoglobinopathies were female (391/618) and 27.7% were males (227/618). Among female, Sickle cell Trait was commonest hemoglobinopathy (220/391), followed by Sickle cell disease (69/391), Thalassemia trait (74/391), S-Beta Double heterozygous (28/391). Among males, Sickle cell Trait was commonest hemoglobinopathy (124/227), followed by Sickle cell disease (55/227), Thalassemia trait (31/227), S-Beta Doubleheterozygous (17/227). The hemoglobinopathies Hb D (1/391) and Hb E (1/391)were observed only in females and Thalassemia major hemoglobinopathy (2/227) was observed among males only (Figure 3).

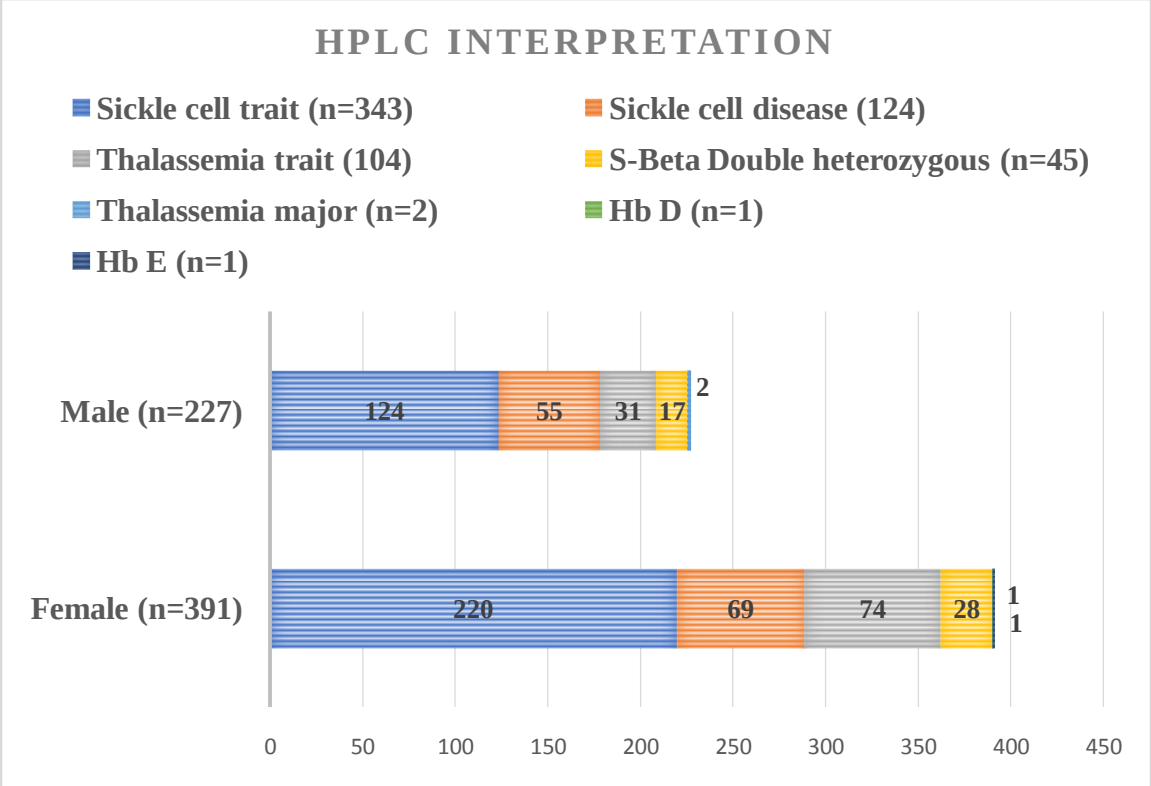

Figure3. Gender wise distribution of hemoglobinopathies variants detected by HPLC among confirmed subjects (N=618).

In the present study the highest mean Haemoglobin was observed in the Thalassemia trait (10.2 ± 2.3 g/dl) and lowest mean haemoglobin was noticed in Thalassemia major (4.8 ± 3.5 g/dl). The mean haemoglobin levels in Sickle cell trait were9.7 ± 3.3 g/dl and in Sickle cell disease were 6.3 ± 1.7 g/dl.Similarly, the highest RBC count was observed in the Thalassemia trait (4.7 ± 0.9x 10^6 mm^3) and lowest RBC count was noticed in Thalassemia major (2.5 ± 1.4x 10^6 mm^3). The mean haemoglobin levels in Sickle cell trait were 3.7 ± 1.5x 10^6 $mm^3$and in Sickle cell disease were 3.3 ± 1.1x 10^6 mm^3. In the present study RDW-CV (%) was raised among subjects with hemoglobinopathies and it was 16.2+5.3% in Sickle cell trait, 22.5 ± 5.7% in Sickle cell disease, 19.4 ± 4.5% in Thalassemia trait, 19.2 ± 1.6% in Double heterozygous for thalassemia and sickle cell, 26.7 ± 6.5% in Thalassemia major, 18.7% in Hb D and 19.2% in Hb E. In the present study MCV (fl) was lowered among subjects with hemoglobinopathies and it was 79.7 ± 13.5 fl in Sickle cell trait, 75.3 ± 11.7 fl in Sickle cell disease, 70.1 ± 9.8 fl in Thalassemia trait, 64.2 ± 5.6 fl in Double heterozygous for thalassemia and sickle cell, 73.1 ± 5.2 fl in Thalassemia major, 79.4 fl in Hb D and 78.2 fl in Hb E. Similarly, MCHC (g/dl) was lowered among subjects with hemoglobinopathies and it was 30.3 ± 2.4 g/dl in Sickle cell trait, 33.6 ± 4.1 g/dl in Sickle cell disease, 29.6 ± 2.6 g/dl in Thalassemia trait, 33.1 ± 0.8 g/dl in Double heterozygous for thalassemia and sickle cell, 28.8 ± 2.5 g/dl in Thalassemia major, 32.7 g/dl in Hb D and 32.6 g/dl in Hb E. The haemoglobin variant Hb A (%) was highest as 89.1 ± 2.7% in Thalassemia trait and lowest as 5.2 ± 10.5% in Sickle cell disease. The haemoglobin variant Hb A2 (%) was highest as 24.5% in Hb D and lowest as 2.5% in Hb E hemoglobinopathies. The haemoglobin variant Hb F (%) was highest as 64.6 ± 28.4% in Thalassemia major and 1.2% in Hb D hemoglobinopathy (Table 1).

Table 1:Haematological parameters and haemoglobin fractions (mean ± SD) in hemoglobinopathies variants detected by HPLC among confirmed subjects (N=618).

Hb variant	Hb (g/dl)	RBC $\times 10^6$ mm^3	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW-CV (%)	HbA (%)	HbA_2 (%)	HbF (%)
Sickle cell trait (n=343)	9.7 ± 3.3	3.7 ± 1.5	79.7 ± 13.5	22.9 ± 5.2	30.3 ± 2.4	16.2+5.3	30.3 ± 2.4	3.4 ± 0.5	2.9 ± 2.4
Sickle cell disease (n=124)	6.3 ± 1.7	3.3 ± 1.1	75.3 ± 11.7	25.8 ± 4.3	33.6 ± 4.1	22.5 ± 5.7	5.2 ± 10.5	3.6 ± 2.5	8.1 ± 5.2
Thalassemia trait (n=104)	10.2 ± 2.3	4.7 ± 0.9	70.1 ± 9.8	21.2 ± 3.5	29.6 ± 2.6	19.4 ± 4.5	89.1 ± 2.7	5.5 ± 0.8	1.2 ± 0.8
S-Beta Double heterozygous (n=45)	8.6 ± 0.9	3.7 ± 0.3	64.2 ± 5.6	21.7 ± 1.7	33.1 ± 0.8	19.2 ± 1.6	42.3 ± 2.4	3.3 ± 0.6	15.6 ± 2.7
Thalassemia major (n=2)	4.8 ± 3.5	2.5 ± 1.4	73.1 ± 5.2	20.1 ± 2.9	28.8 ± 2.5	26.7 ± 6.5	23.6 ± 22.5	4.1 ± 1.3	64.6 ± 28.4
Hb D (n=1)	9.7	4.1	79.4	25.1	32.7	18.7	62.4	24.5	1.2
Hb E (n=1)	10.2	4.2	78.2	25.9	32.6	19.2	53.5	2.5	1.6

DISCUSSION

HPCL has been proven to be a sensitive, specific, and repeatable alternative to electrophoresis. It promises being a precise and reliable method in rapid detection and characterization of physiologic and pathologic haemoglobin fraction with automated and analytical capacity [9,10,11,12,13,14]. The accuracy of retention intervals acquired using preserved normal [14] and abnormal samples [11,12] has been the subject of several studies. A few Indian studies [15,16] investigated and underlined the usefulness of HPLC in the detection for β - thalassemia as well as other haemoglobinopathies.

In the β-thalassemia trait group, RDW-CV was greater (19.4 ± 4.5%) in the present study than in the studies of Aslan et al. (14.88 percent), Rathod et al. (14.47 percent), and Demir et al. (14.91 percent). This matched the severity of anisopoikilocytosis in the present study subject. It could be due to the fact that in the present study, simultaneous iron insufficiency was present in 14.7 percent of subjects. Iron insufficiency has been linked to a reduction in HbA2 levels [17]. β -thalassemia trait can usually be detected in the presence of iron insufficiency. Madan et al. [18] looked at the iron status of 463 heterozygous beta-thalassaemic and found that 27.2 percent of them were iron deficient and in that study the two groups of patients with the characteristic had similar mean HbA2 levels, and all but one heterozygote tested had increased HbA2 levels (>3.5%).

In present study, the thalassemia trait, and thalassemia major patients, MCV (70.1 ± 9.8fl and 73.1 ± 5.2fl respectively) and MCH (21.2 ± 3.5pg and 20.1 ± 2.9pg respectively) proved to be nearly identical. The thalassemia trait was associated with a high red cell count (4.7 ± 0.9x 10^6 mm^3). Though anisopoikilocytosis was minimal in thalassemia trait, it was moderate to severe in thalassemia major. On the basis of red cell morphology alone or absolute values, it looks difficult to distinguish between thalassemia major and intermedia. As a result, in order to make an appropriate diagnosis, blood results must be compared to the clinical picture. In certain tough situations, the percentage of Hb and/or the band on electrophoresis assisted in the diagnosis[19,20].

In present study, 945 subjects were screened for hemoglobinopathies and it was observed that 36.3% of subjects had Sickle cell trait (343/945), 13.1% had Sickle cell disease (124/945), 11.0% had Thalassemia trait (104/945), 4.7% had S-Beta Double heterozygous (45/945). Thalassemia major (2/945), Hb D (1/945) and Hb E (1/945) were observed among 0.2%, 0.1% and 0.1% of subjects respectively. The 34.6% of subjects (317/945) were having no hemoglobinopathies. In a study by Campbell et al. [21], a total of 25750 samples were evaluated using HPCL and 95.5% of the samples were found normal, and 4.5% of the samples had hemoglobinopathies, with 48.8% of the abnormal samples showing Sickle cell trait hemoglobinopathy. In a study by Sachdev et al. [22], a total of 2600 samples were evaluated using HPCL and 87.4% of the samples were found normal, and 12.6% of the samples had hemoglobinopathies, with 70.9% of the abnormal samples showing Beta thalassemia trait hemoglobinopathy. In a study by Rao et al. [23], a total of 800 samples were evaluated using HPCL and 69.1% of the samples were found normal, and 30.9% of the samples had hemoglobinopathies, with 58.7% of the abnormal samples showing Beta thalassemia trait hemoglobinopathy. In a study by Chandrashekar et al. [24], a total of 543 samples were evaluated using HPCL, with 37.9% of abnormal samples showing Beta thalassemia trait hemoglobinopathy. In a study by Bhalodia et al. [25], a total of 500 samples were evaluated using HPCL and 91.4% of the samples were found normal, and 8.6% of the samples had hemoglobinopathies, with 60.5% of the abnormal samples showing Beta thalassemia trait hemoglobinopathy. In a study by Pant et al. [26], a total of 4800 samples were evaluated using HPCL and 94.0% of the samples were found normal and 6.0% of the samples had hemoglobinopathies with 74.5% of the abnormal samples showing Beta thalassemia trait hemoglobinopathy. In a study by Mondal et al. [27], a total of 119336 samples were evaluated using HPCL and 87.8% of the samples were found normal, and 12.2% of the samples had hemoglobinopathies with 37.8% of the abnormal samples showing Beta thalassemia trait hemoglobinopathy. In a study by Banerjee et al. [28], a total of 1048 samples were evaluated using HPCL and 42.4% of the samples were found normal, while 57.6% of the samples had hemoglobinopathies with 25.8% of the abnormal samples showing Beta thalassemia trait hemoglobinopathy.

The current findings demonstrate that HPLC is an efficient and reliable diagnostic technique for the easy detection of haemoglobin variations and the characterization of normal and abnormal haemoglobin components with a high level of precision. CE-HPLC (β-thal short program) could be a useful technique for quickly diagnosing a wide range of haemoglobinopathies. The retention time and proportion of variant haemoglobin can help distinguish variant haemoglobins that elute in the same window. HbA2 levels are significantly lower in iron-deficient anaemia. HbA2 in the borderline range should be investigated further, particularly for silent mutations, α- thalassaemia, and co-existing nutritional deficiencies.

According to current standards, abnormal variant Hbs must be validated using a different method. This is good practise, because it's usually cheap and simple (like a sickling test for S-window peaks, or electrophoresis for others). It's especially crucial when screening pregnant women because the diagnosis can affect prenatal testing.

CONCLUSION

Nutritional deficiencies, which can be remedied with drugs, are the leading cause of anaemia in India. Anemia caused by abnormal haemoglobin should also be evaluated, as morbidity and mortality are significant in homozygous haemoglobinopathies. Haemoglobin anomalies are mostly limited to specific regions, faiths, castes, and tribes, and by understanding the prevalence, we can raise awareness about the diseases and their consequences among the general public as well as take actions to treat them and aid in prevention. In the detection of

haemoglobinopathies, automated cation exchange HPLC is gradually being used as the primary diagnostic test. It is a precise, easy, and better technique for detecting various haemoglobin abnormalities, which aids in patient care and has prognostic value.

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