A STUDY OF HIGHPERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) IN PATIENTS PRESENTING WITH ANAEMIA AT A TERTIARY CARE HOSPITAL

AVANI DANGAR[1], PALAK PATEL[1], MIRAT DONGA[1], KRISHNA KANT SHIROMANI[2] AND KIRAN DELWADIA[2]

Department of Pathology, Parul Institute of Medical Sciences and Research, Vadodara, Gujarat, India

Abstract– The most prevalent type of genetically inherited red blood cell disorder worldwide are hemoglobinopathies. Screening and accurate identification of hemoglobin (Hb) variants have become increasingly important in antenatal diagnosis and prevention of Hb disorders. The objective is to identify haemoglobin abnormalities by high performance liquid chromatography (HPLC) for screening the anaemic patients who are visiting the hospital in OPD and admitted in IPD. This is cross sectional retrospective study for a period of 6 months and performed in a tertiary care hospital on patients presenting with anemia in different Departments. In total, high performance liquid chromatography HPLC of 400 patients with anemia was studied. Out of 400 patients, 190 were males and 210 were females. Age group varied from infants to middle-aged adults. The following criteria were used to identify hemoglobinopathies on HPLC patterns as follow: HbS:- 10%-45%: Sickle cell trait, 45%-50%: HbS>HbA-Sickle cell disease, HbS<HbA-Sickle cell trait, >50%: Sickle cell disease, HbA2:- >3.5%: Beta thalassemia trait, HbA2 normal or increase and HbF increase upto 90% : Beta thalassemia major, HbF:- 5% to 30%: hereditary persistence of fetalhemoglobin, HbD Variant: Retention time of unknown peak between 3.90-4.30 minutes. Age and sex was expressed in actual number and percentages. Countinuous variables were presented as mean ± 2SD. Chi square statistics were used to compare categorical variables. P<0.005 was statistically significant. Microsoft excel and graph pad calculator was used for data analysis. 55% cases (total = 220) having hemoglobinopathies. Among those with hemoglobinopathies 120 (55%) were females and 100 (45%) were males. Most of the patients being less than 30 years. HPLC results include 19.75% cases of sickle cell disease, 1.5% of sickle-beta thalassemia, 27.25% of sickle cell trait, 4.5% of beta thalassemia trait, 0.5% of beta thalassemia major, 0.5% of HbD variants, 0.5% of hereditary persistence of fetal hemoglobin and 0.5% of unknown peaks, they have been listed as others. HPLC is very simple, accurate and superior technique in timely detection of various haemoglobin disorders, which helps in early management of patients.

INTRODUCTION

Hemoglobinopathies are a group of hereditary disorders characterized by structural alterations of hemoglobin molecule, that result from either production of abnormal hemoglobin chains, such as substitution of one amino acid as seen with Sickling disorders or underproduction of a given globin chain as in the thalassemias *(Wilson et al., 2010).*

Hemoglobinopathies are a global problem and associated with numerous morbidities and mortalities in developing countries. World Health Organization (WHO) recognized about 7% of the world populations are carrier for different hemoglobin disorders and about 80% of the affected children are born in developing countries (Nicosia and Cyprus, 2007). In India, Sickle cell disease range is varying within various communities from 1% to 44% and â-Thalassemia is prevalent across the country, with an average frequency of carriers being 3-4%. (Madan N *et al.*, 2010, Sinha *et al.*, 2009, Mohanty *et al.*, 2013). Evaluation of patients and carriers of hemoglobinopathies is done by RBC indices and peripheral smear examination followed by examination of Hb pattern on HPLC or

([1]PG Resident, [2]Professor)

electrophoresis. Hemoglobin fraction analysis by cation-exchange HPLC is the commonest test used worldwide for secondary screen or laboratory diagnosis of hemoglobinopathies. It provides percentage quantification of different Hemoglobin fractions.

Timely diagnosis and proper management would reduce the mortality rates due to hemoglobinopathies and improve the clinical outcome. It is therefore necessary to conduct studies to determine the frequency of hemoglobinopathies so as to increase the awareness of the extent of the problem. This study has thus been carried out to study the high performance liquid chromatography (HPLC) of patients who presented with anaemia in a tertiary care hospital, to determine the frequency and demographic distribution of hemoglobinopathies in these patients.

METHODS

This is cross sectional retrospective study for a period of 6 months from July to december 2022 was performed in a tertiary care hospital on patients presenting with anemia in different departments. In total, High performance liquid chromatography (HPLC) of 400 patients with anemia were studied. Out of 400 patients, 190 were males and 210 were females. Age group varied from infants to middle-aged adults.

Study Design: The patients whose hemoglobin concentrations fell in the range of mild to severe anemia, according to WHO recommendations (WHO, UNICEF, and UNU, 2001) and were not having any other chronic illness were included in the study (Table 1). High Performance Liquid Chromatography to identify variant and abnormal hemoglobin.

Table 1. WHO Cut Off for Severity of Anaemia

Age	Mild	Moderate	Severe
6-59 Months	10.0-10.9	7.0-9.9	<7.0
5-11 Years	11.0-11.9	8.0-10.9	<8.0
12-14 Years	11.0-11.9	8.0-10.9	<8.0
Male >14 Years	11.0-11.9	8.0-10.9	<8.0
Female >14 Years (non-pregnant)	11.0-11.9	8.0-10.9	<8.0
Female >14 Years (pregnant)	10.0-10.9	7.0-9.9	<7.0

The following criteria were used to identify hemoglobinopathies on HPLC patterns:

HbS:- 10%-45%: Sickle cell trait, 45%-50%: HbS>HbA-Sickle cell disease, HbS<HbA-Sickle cell trait, >50%:Sickle cell disease (Alla Joutovsky et al., 2004)

HbA2:->3.5%: Beta thalassemia trait (Alla Joutovsky et al., 2004)

HbF: 5-30%: hereditary persistence of fetalhemoglobin (Alla Joutovsky et al., 2004)

HbD Variant:- Retention time of unknown peak between 3.90-4.30 minutes (Alla Joutovsky et al., 2004)

HbA2 normal or increase and HbF increase upto 90%:- Beta thalassemia major (Alla Joutovsky et al., 2004)

Statistical Analysis

Age and sex was expressed in actual number and percentages. Continuous variables were presented as mean ± 2SD. Chi square statistics were used to compare categorical variables. $P<0.005$ was statistically significant. Microsoft excel and graphpad calculator was used for data analysis.

Ethics

This was cross-sectional observational study and no intervention was done. Approval from Institutional Ethics Committee was obtained.

RESULTS

Out of the 400 samples tested for haemoglobin abnormalities, 220 (55%) were found to have hemoglobinopathies (Figure 1).
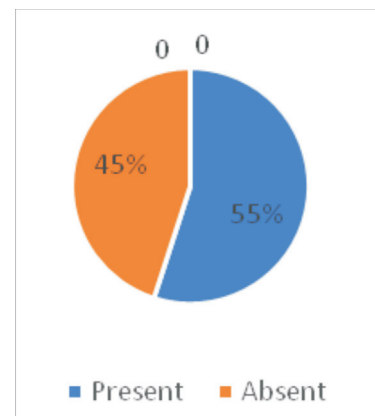

Fig. 1. Prevalence of homoglobinopathies

Most of the patients who were diagnosed with hemoglobinopathy were less than 30 years of age, with majority (88 cases) of them being in the age group of 20-29 years. 37 patients were more than 30 years of age (Figure 2).
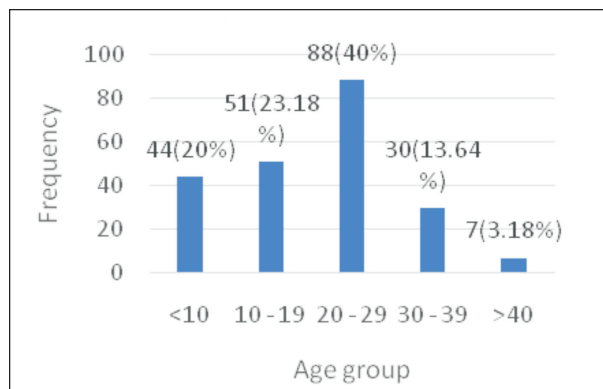
Fig. 2. Age wise frequency distribution of homoglobinopathies

Among those with hemoglobinopathies (total = 220), 120 (55%) were females and 100 (45%) were males (Figure 3).

Among the 190 males tested, 100 were positive for hemoglobinopathies while among 210 females tested, 120 were positive for hemoglobinopathies.

Out of all the cases studied, 180 (45%) were normal, i.e., no hemoglobinopathy detected. The most common hemoglobinopathies detected were sickle cell disorders (including both trait and disease) being 188 (47%), followed by Beta thalassaemia (including both trait and major) – 20 (5%), 6 (1.5%) cases of HbS with coexistent Sickle-beta thalassemia, 2 (0.5%) cases of HbD variant and 2 (0.5%) cases of Hereditary persistence of fetal haemoglobin (HPHF). Hb Electrophoresis of 2 (0.5%) cases showed unknown peaks and have been listed as others (Figure 4).
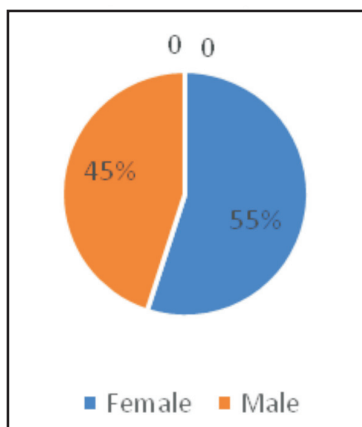

Fig. 3. Gender distribution of homoglobinopathies

Patients diagnosed to have Beta thalassemia major having an average haemoglobin concentration of 4.85 g/dL. Those with sickle cell disease had an average Hb of 5.13 g/dL, those with Sickle-Beta thalassemia had average haemoglobin of 6.5 g/dL, those with Beta thalassemia trait had an average haemoglobin concentration of 9.8 g/dL and those with sickle cell trait had an average haemoglobin concentration of 10.35 g/dL. Patient with HbD trait had haemoglobin 9.4 g/dL and one with HPHF had haemoglobin 11.6 g/dl.
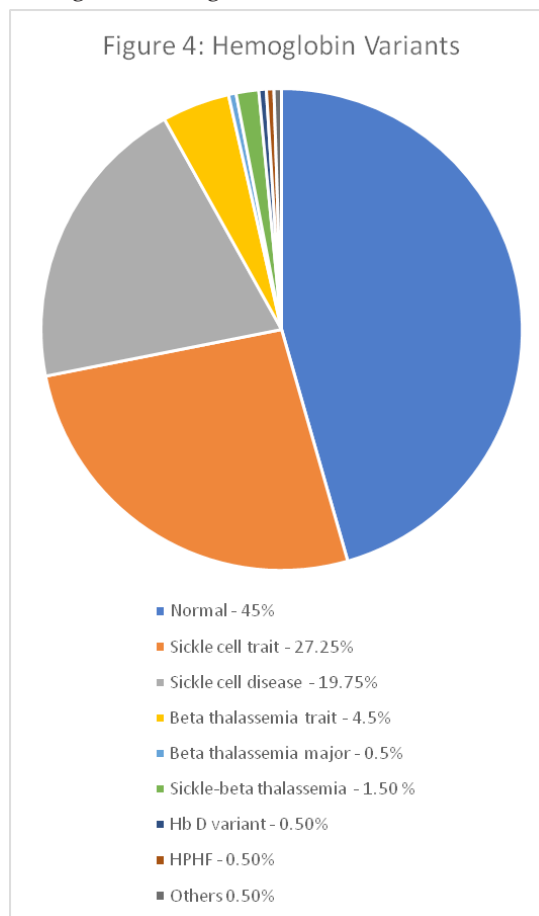
Among all patients with hemoglobinopathies most cases (47%) presented with moderate anaemia which has been closely followed by severe anaemia (44% cases) and the least number of cases (9%) with mild anaemia (Figure 5).

To establish an association between two qualitative variables, whether they are statistically significant or not, the chi-square test: a test of significance is to be conducted. In our study, to establish an association between gender and hemoglobinopathies, formulate a cross-table. The chi-square statistic compares observed values to the expected values, where observed values are the actual counts obtained from the sample and the expected values specify what the values of each cell

of the table would be if there was no association between two variables.

$$\text{Expected value} = \frac{\text{Row total and Column total}}{\text{Table total}}$$

$$\chi^2 = \Sigma \ \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

Values were entered into the formula for the chi-square statistic, and the chi-square value obtained was 0.819. The final step of chi-square test of significance is to determine if the value of the chi-square test statistic is large enough to reject the null hypothesis.

The p-value for the chi-square statistic is 0.36, which is larger than the alpha level p-value 0.05. Therefore, accepting null hypothesis, evidence from the study shows that there is no significant difference between gender and hemoglobin opathies.

DISCUSSION

In this study, 55% cases (total=220) having abnormal Hb variants were detected, of which majority were less than 30 years of age. Among those with hemoglobinopathies 120 (55%) were females and 100 (45%) were males. In comparison, the study conducted by Khanam *et al*. (2020) showed 43% cases with abnormal Hb variants with most of the patients being less than 20 years of age, of which 40% were females and 60% were males.

Sickle cell disease (SCD) is a protean disorder with incidence range in India, varying within various communities from 1% to 44%.[9] It leads to hemolytic anemia, acute vasoocclusion, and organ damage due to recurrent erythrocyte sickling. Our HPLC results include 19.75% cases of Sickle cell disease, 1.5% with Sicklebetathalassemia and 27.25 % with Sickle Cell Trait. In contrast, Khanam *et al.* (2020) records 23% cases of Sickle-beta-thalassemia, 10% cases of Sickle cell trait and 8% cases of Sickle cell disease.

The most prevalent singlegene Hb diseases worldwide are alpha- and betathalassemia. The diverse castes and tribal groupings that make up the Indian population each exhibit distinctive genetic characteristics. Betathalassemia is more prevalent in some communities in India, including Sindhi, Gujarati, Punjabi, and Bengali (incidence ranges from 1% to 17%) (Gupta *et al*., 2003) study

carried out by Christianson *et al*. (2006). 1.2/ 1000 live births are thought to be the prevalence of abnormal hemoglobinopathies in India. One of the greatest techniques for screening, detecting, and identifying a variety of hemoglobinopathies with quick, repeatable,and accurate results is HPLC (Bravo Urquiola, 2004). In our study include 4.5% of Beta-thalassemia trait. In contrast, studies by Warghadeet *et al*., 2018; Sachdev *et al*., 2010 and Bhalodia *et al*., 2015 showed similar results (11.21%, 8.9% and 1.2%) with most cases of those with abnormal Hb, being that of Beta thalassemia trait. In contrast, study by Khanam *et al*., (2020). had the majority 23% of abnormal Hb variant being Sickle-Beta thalassemia. Our study also records 0.5% cases of beta thalassemia major. Although betathalassemia major affects people all over the world, it is more common in the Mediterranean region, Middle East, South East Asia, South West Europe, and Central Africa (Aziz *et al*., 2012). It is caused by a mutation in the betaglobin gene (HBB) on chromosome number11.

0.5% cases with HbD heterozygous have also been recorded in this study. Homozygous HbD/ HbDbetathalassemia and heterozygous HbD accounted for 0.09% and 0.48% cases, respectively, in the study by Warghade (Warghade *et al*., 2018). In northwest regions of India HbD is very common. HbDbetathalassemia is more prevalent and more dangerous than homozygous HbD. Homozygous HbD is unusal and menifests as a relatively milder type of disease. Double heterozygosity of HbD with HbS leads to anemia which is moderate to severe form of disorder.

HPFH was first documented in Ghana (Bravo Urquiola *et al*., 2004) and has also been described in nonAfrican populations. Adult erythroid cells in HPFH express the gammaglobin gene of Hb F at high quantities.Our study reported 0.5% cases of HPFH.

0.5% cases in the study showed one with high HbA2 levels (3.8%) and one with indeterminate HbA2 levels (3.4%), inconclusive HPLC reports elevated Hb F (7.7%), Hb A2 (2%) and Hb A0 (69.3%) with multiple unknown peaks which have been listed as others.

According to research conducted in India, prenatal diagnosis is still the best course of action for preventing the delivery of the homozygous children. Prenatal testing detects asymptomatic individuals whose children are at risk of inheriting the condition of hemoglobinopathies. With the use

of this knowledge, parents might make informed decisions about having children and due to which non immune hydrops fetalis pregnancy might be prevented. Premarital counselling and screening can be applied to pregnant women/individuals from a high risk ethnic backgrounds and consanguineous marriages (Warpe *et al*., 2016)

CONCLUSION

As determined by the study, there is a considerable chance that patients presenting with anaemia can have some underlying genetic/hereditary disorder instead of nutritional causes of anaemia. Therefore, steps are needed to be taken for proper diagnosis and management of patients with anaemia in order to reduce burden of the disease as well as cost of treatment and general outcome of the patient. It is also necessary to educate people regarding these conditions and to encourage premarital genetic counselling for prevention and a better approach at dealing with these disorders.

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